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(54) Title: USE OF ORAL TOLERANCE TO SUPPRESS BOTH Th1 AND Th2 IMMUNE RESPONSES AND TO SUPPRESS ANTIBODY PRODUCTION

(57) Abstract

This invention relates to methods for orally administering autoantigens to suppress specific Th2 as well as Th1 immune responses and antibody production. The invention finds applicability in the treatment of antibody-mediated autoimmune diseases.

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USE OF ORAL TOLERANCE TO SUPPRESS BOTH Th1 AND Th2 IMMUNE RESPONSES AND TO SUPPRESS ANTIBODY PRODUCTION

Field of the Invention

This invention relates to methods for suppression of specific Th2 (as well as Th1) immune responses and antibody production and finds applicability in the treatment of antibody-mediated autoimmune diseases.

Background of the Invention

The use of the oral route to tolerize the immune system against antigens (foreign or self) has been employed to suppress immune response associated with T-cell mediated human autoimmune diseases (1,2). Antigen feeding as a means to generate peripheral tolerance in CD4+ cells has been thus far successful in tolerization of Th1 type responses, while those of Th2 lymphocytes appear to be intact (3-5). Maintenance of Th2 responses has value in regulating Th1 responses during the induction of oral tolerance to Th1-mediated immune diseases (bystander suppression). In antibody-mediated autoimmunity, however, Th2 responses play an important pathogenetic role. It would be therefore desirable to suppress Th2 autoimmune responses associated with antibody-mediated autoimmune diseases. However, up to the present time, Th2 suppression has not been induced as the result of orally-induced tolerance.

There have been several reports (4, 19, 34, 35) that oral administration of single doses and relatively high amounts of antigen in experimental models of Th1-mediated autoimmune disease has induced T-cell anergy, especially anergy of Th1 responses. Anergy is a state of antigen-specific T-cell

nonresponsiveness. However, the degree to which anergy contributes to Th2 tolerance is still under investigation.

Difficulties in generating tolerance of Th2 lymphocytes *in vitro* (6-8) or of Th2-mediated antibody production *in vivo* have been encountered in other experimental systems in which tolerance was induced by intravenous (I.V.) or intraperitoneal (I.P.) administration of soluble antigens (9-12). Thus, after tolerization by the oral or parenteral route, IL-2 and IFNγ were not produced in cultures, and a diminished IgG2a antibody response was observed *in vivo* (tolerance of Th1 responses). In contrast, IL-4 production *in vitro* and IgG1 responses *in vivo* were intact (intact Th2 responses) (3-11). It appeared, therefore, that Th2 lymphocytes were resistant to tolerance induction, whether by oral or parenteral route.

B-cells have been even more resistant to suppression by tolerization techniques.

On the other hand, the oral route of exposure to antigen has evolved as an efficient pathway to generate peripheral tolerance to food antigens (13). As a result, both cell-mediated immunity (controlled by Th1 lymphocytes) and hypersensitivity (controlled by Th2 lymphocytes) to food antigens appear to be prevented in normal subjects.

In light of this, the present inventors endeavored to discover oral tolerance techniques that would cause suppression of specific Th2 lymphocytes. Since Th2 lymphocytes play an important role in antibody production, successful suppression of Th2 responses would prove a useful tool in suppressing abnormal antibody-mediated immune responses.

Oral tolerance is a clinically attractive method to treat immune dysfunctions (such as autoimmune diseases) for several reasons:

(1) Absence of toxicity - No toxicity has been observed in clinical trials involving oral administration of bovine myelin (which contains MBP and PLP) to humans afflicted with multiple sclerosis; or oral administration of chicken Type II collagen to humans afflicted with rheumatoid arthritis; or oral administration of bovine S-antigen to humans afflicted

with uveoretinitis; or oral administration of insulin to healthy volunteers.

(2) Avoidance of global immunosuppression - Previously available treatments involved the administration of steroids, or cyclosporine A, or chemotherapeutic drugs or biologic response modifiers and other global immunosuppressive agents which diminished the ability of the treated subject to defend against pathogens (and was accompanied by various other known side effects ranging from unpleasant to life-threatening). Oral tolerance on the other hand would accomplish specific suppression of abnormal immune responses.

(3) Convenience of therapy.

Oral tolerance to autoantigens (defined solely for purposes of this background discussion as antigens that are primary targets of attack by the immunoregulatory system) and bystander antigens (briefly, antigens specific to the organ or tissue affected in a T-cell mediated autoimmune disease but not necessarily a target of autoimmune attack) has been induced successfully by daily (and/or less frequent) administration of such antigens and has been employed to suppress Th1-mediated autoimmune reactions or responses, and thus to suppress (T-cell mediated) autoimmune disease in both animals and humans: Thompson, H.S.G. et al. *Clin. Exp. Immunol.*, *64*:581-586, 1986; Nagler-Anderson, C. et al. *Proc. Nat'l. Acad. Sci. (USA) 83*: 7443-7446, 1986; Higgins, P. et al. *J. Immunol. 140*: 440-445, 1988; Zhang, J.A. et al. *Proc. Natl. Acad. Sci. (USA) 88*:10252-10256, 1991; Nussenblatt, R.B. et al., *J. Immunol.*, 144:1689-1695, 1990; Weiner, H.L. et al. *Science, 259*:1321-1324, 1993; Trentham, D.E. et al., *Science*, 261, 1727-1730, 1993. In the case of Th1-mediated autoimmune disease, oral tolerance results in active suppression, i.e., elicitation of antigen specific T-cells which are or include Th2 cells and which are targeted to the afflicted tissue and exert local suppressive effect.

Methods and compositions useful in suppression of an immune response associated with a T-cell mediated or T-cell dependent autoimmune disease by orally induced tolerance (or by tolerance induced by inhalation) using daily or less

frequent administration of autoantigens or more generally bystander antigens with and without enhancers have been described in various patents and patent applications by the present inventors and their co-workers: Ser. No. 07/843,752, filed February 28, 1992; Ser. No. 08/202,677, filed February 25, 1994; Ser. No. 08/419,502, filed April 10, 1995; Ser. No. 08/419,505, filed April 10, 1995; Ser. No. 08/235,121, filed April 28, 1994; Pat. No. 5,399,347, issued March 21, 1995; Ser. No. 08/297,395, filed August 11, 1994; Ser. No. 08/046,354, filed April 9, 1993; Ser. No. 08/420,979, filed April 10, 1995; Ser. No. 08/420,980, filed April 10, 1995; Ser. No. 08/105,912, filed August 10, 1993; Ser. No. 08/279,275, filed July 22, 1994; Ser. No. 08/328,562, filed October 24, 1994. These techniques induce suppression of Th1 responses, which is beneficial in the treatment of T-cell mediated or T-cell dependent autoimmune diseases. The Th2 responses are not suppressed and are relied upon to regulate Th1 responses (bystander suppression).

It would nevertheless be desirable to devise methods to extend the use of orally-induced tolerance to the treatment of other immune dysfunctions. One such class of immune dysfunctions are antibody-mediated autoimmune diseases, (for example certain aspects of systemic lupus erythematosus, autoimmune thyroiditis, myasthenia gravis, glomerulonephritis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, pemphigus vulgaris, Grave's disease, insulin resistance (encountered in Type II diabetes), and pernicious anemia).

Objects of the Invention

It is an object of this invention to devise methods and compositions for suppressing Th2 autoimmune responses (alone or together with Th1 responses) via the oral route.

Another object is to devise methods and compositions for suppressing abnormal antibody-mediated immune responses.

A further object is to devise methods and compositions for treating antibody-mediated autoimmune

responses, in order to treat antibody-mediated autoimmune diseases.

Summary of the Invention

In one aspect the present invention relates to a method for treating a mammal suffering from an antibody-mediated autoimmune disease comprising orally administering to said mammal at least one autoantigen (as defined below) specific for said disease; and continuing said administration for a period of time until a Th2 cell mediated autoimmune response associated with said disease is suppressed. The amount of said antigen, the schedule (frequency) of said administration, and the period of time are selected to effect said suppression.

In another aspect, the present invention relates to a method for treating a mammal suffering from an antibody-mediated autoimmune disease comprising administering to said mammal via the oral route an autoantigen specific for said disease for a period of time sufficient to accomplish at least one of the following: reduce the number of autoreactive Th2 cells in said mammal recognizing said autoantigen; reduce the number of autoreactive antibodies in said mammal recognizing said autoantigen; and eliminate or decrease the severity of at least one clinical symptom or indicator associated with said disease.

In a further aspect, the present invention relates to a method for treating a mammal suffering from an antibody-mediated autoimmune disease comprising parenterally administering to said mammal at least one autoantigen specific for said disease; and continuing said administration until a Th2 cell mediated autoimmune response associated with said disease is suppressed.

Other aspects of the invention relate to suppression of a Th2 (or both Th2 and Th1) response associated with antibody-mediated autoimmune disease by oral administration of at least one autoantigen specific for the antibody-mediated autoimmune disease in an amount, a frequency of administration and for a period of time effective to suppress said response.

Further aspects of the invention will be apparent to those skilled in the art in light of the present description, and accompanying claims and drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are plots of spleen cell proliferation averages ± SEM in absorbance units at 570-630nm against stimulating antigen concentration *in vitro*. Mice were continuously exposed to OVA in drinking water for 20 days and primed by OVA-CFA (open triangles). Control mice were primed by OVA-CFA (filled circles) or CFA alone (open circles). Pooled erythrocyte depleted spleen cells were prepared 10 days after immunization and stimulated by different concentrations of OVA (Fig. 1A) or PPD (Fig. 1B).

Figures 2A, 2B, 2C and 2D are plots of antibody titers (averages ± SEM) from individual mice (for all isotypes) expressed in absorbance units at 405 nm against reciprocal serum dilution. Mice were continuously fed with OVA (open triangles). Control mice were primed by OVA-CFA (filled circles) or by CFA alone (open circles). Serum samples were collected 15 days after immunization and individually assayed for IgG2a (Fig. 2A), IgG2b (Fig. 2B), IgG1 (Fig. 2C) and IgE (Fig. 2D).

Figure 3 is a plot of OVA specific cytokine release or antibody titers v. amount of fed antigen. Mice were continuously fed with different dosages of OVA for 20 days and primed by OVA-CFA. Cytokine (circles) and antibody (triangles) secretion respectively are shown for IL-2 (solid circles) and IL-4 (open circles). Cytokine results are averages ± SEM of quadruplicate cultures and are expressed in absorbance units at 570-630nm. IgG2a (solid triangles) and IgG1 (open triangles) are also shown. Antibody titers are averages ± SEM of titers from individual mice and are expressed in absorbance units at 405nm.

Figure 4 is a plot of average antibody binding ± SEM from individual mice at a serum dilution of 1:400, presented in absorbance units at 405 nm against time of exposure to anti-

gen. IgG1 are shown by open circles and IgG2a are shown by solid circles.

Figure 5A is a plot of the percentage of Vβ8.2+ T-cells which are also CD4+ versus antigen (OVA) feedings over feeding frequency. Mice were fed 0.5 (open circles), 5 (filled squares) or 500 mg (filled circles) of OVA every other day for five feedings, and T-cells were harvested for analysis prior to, and 24 hours after, each feeding.

Figures 5B and 5C are fluorescence contour plots showing the probability of incidence of T-cells of various subtypes; CD4+ cells are depicted in the upper right-hand quadrant.

Figure 6A is a plot of the percentage of Vβ8.2+ cells which are undergoing apoptosis versus antigen (OVA) feedings over feeding frequency. Vβ8.2+ cells from mice fed 0.5 (filled circles), 5 (filled squares), and 500 mg (closed circles) were analyzed for the presence of degraded DNA which indicates apoptosis by staining with acridine orange.

Figures 6B and 6C are Forward Angle Light Scatter (FALS) plots showing the incidence of acridine orange staining (indicating dying cells) for various T-cell subtypes.

Figure 7A is a plot of percentage of Vβ8.2+ cells which are actively dividing versus antigen (OVA) feeding frequency. Vβ8.2+ cells from mice fed 0.5 (open circles), 5 (filled squares), and 500 mg (filled circles) were analyzed for DNA content which indicates active division, by staining with propidium iodide.

Figures 7B and 7C are FALS plots showing the incidence of DNA content for various T-cell subtypes from control(B) and OVA-fed animals.

Figure 8A-8E are plots showing the concentrations of cytokines IL-2 (8A), IL-4 (8B), IL-10 (8C), (all in pg/ml) and IFN-γ (8D) and TGF-β (8E)(both in ng/ml) versus antigen (OVA) feeding frequency.

Detailed Description of the Invention

The following terms, when used in this disclosure, shall have the meanings ascribed to them below:

"Mammal" is defined herein as any warm-blooded higher vertebrate organism (including a human) having an immune system and being susceptible to an autoimmune disease.

"Autoimmune disease" is defined herein as a spontaneous or induced malfunction of the immune system of mammals, including humans, in which the immune system fails to distinguish between foreign immunogenic substances within the mammal and/or autologous substances and, as a result, treats autologous tissues and substances as if they were foreign and mounts an immune response against them. The term includes human autoimmune diseases and animal models therefor.

"Autoantigen" is any substance or a portion thereof normally found within a mammal that invokes an immune response within an individual, i.e. that is recognized by activated T-cells of the mammal or by antibodies in the mammal. In an autoimmune disease, such an antigen may be or may become the primary (or a primary) target of attack by the immunoregulatory system. The term also includes antigenic substances that induce conditions having the characteristics of an autoimmune disease when administered to mammals. Additionally, the term includes peptidic subclasses consisting essentially of immunodominant epitopes or immunodominant epitope regions of autoantigens. Immunodominant epitopes or regions in induced autoimmune conditions are fragments or portions of an autoantigen that can be used instead of the entire autoantigen to induce the disease. In humans afflicted with an autoimmune disease, immunodominant epitopes or regions are fragments or portions of antigens specific to the tissue or organ under autoimmune attack and recognized by a substantial percentage of autoimmune attack T-cells or antibodies from a patient or a group of patients. See, e.g., 08/426,784 for determination of T-cell immunodominant epitopes. Autoantigens and their immunodominant epitopes that elicit antibodies can be identified by antibody binding tests, ELISA assays or dot blot analysis using whole antigens or peptide fragments of a particular autoantigen (overlapping peptide method).

"Treatment" of an autoimmune disease is intended to include both treatment to prevent or delay the onset of an

autoimmune disease (or to prevent or delay the manifestation of clinical or subclinical, e.g., histological, symptoms thereof), as well as therapeutic suppression or alleviation of symptoms after the manifestation of such autoimmune disease. In either case, treatment is accomplished by abating autoimmune attack and preventing or slowing down autoimmune tissue destruction. "Abatement", "suppression" or "reduction" of autoimmune attack or reaction encompasses partial reduction or amelioration of one or more symptoms of the attack or reaction, i.e. reduction in number of activated autoreactive T-cells or in number of autoreactive antibodies. A "substantially" increased suppressive effect (or abatement or reduction) of autoimmune reaction means a significant decrease in one or more markers or histological or clinical indicators of autoimmune reaction or disease. Nonlimiting examples of symptoms associated with various autoimmune diseases are given below. In each case an improvement in one or more symptoms reported by the patient (e.g. fatigue) or observed by the attending physician or determined by quantitative or semiquantitative techniques can be used to assess efficacy of treatment according to the invention.

DISEASE	SYMPTOMS/DIAGNOSTIC MARKERS
Autoimmune hemolytic anemia	- anemia - splenomegaly - spherocytosis of blood smears - polychromatophilia with high MCHC - "warm reading" positive direct antiglobulin test (DAG)
Infertility due to Sperm Antigen Auto-antibodies	- autoantibodies to sperm detected by agglutination and sperm immobilization (gelatin, tube-slide, slide, tray, or capillary tube agglutination test) - passive hemagglutination assay (Mathur et al., J. Immunol. Methods 1979, 30:381-393) - radiolabel antiglobulin test (Haas, et al NEJM, 1980, 303:722-727)
Sjögren's Syndrome (SS)	- autoantibodies to SS in serum and saliva - rheumatoid factors - inflammation of salivary glands and lachrymal glands

DISEASE	SYMPTOMS/DIAGNOSTIC MARKERS
Systemic lupus erythematosus	- anemia - fatigue - malar rash - discoid rash - oral ulcers - arthritis - serositis - neurological disorder measured by abnormal electroencephalogram or elevated protein levels in cerebrospinal fluid - autoantibodies to antinuclear antigens determined by immunoassay taken in the absence of drugs known to induce antinuclear antigens. - renal disorder measured by deposit of immunoglobulins, proteinuria, or cellular casts.
Myasthenia gravis	- autoantibodies to acetylcholine receptor determined by immunoassay (preferred marker) - ocular muscle weakness - fatigue - anticholinesterase response - electrophysiological tests to measure response to nerve stimulation - muscle strength (preferred marker)
Autoimmune thrombocytopenic purpura	- antibodies to platelets
Primary Biliary cirrhosis	- serum alkaline phosphatase increase - bilirubin increase - IgM and IgG increase - hyperlipidemia - lipoprotein X - serum bile salts increased
Ulcerative colitis	- improvement assessed by reduction in fever, decreased bloody diarrhea and improvement in appetite
Wegener's Granulomatosis	- anemia - sedimentation rate - rheumatoid factor - uremia
Insulin resistance	- measurement of anti-insulin antibodies - ketoacidosis - hyperglycemia - hyperinsulinemia

DISEASE	SYMPTOMS/DIAGNOSTIC MARKERS
Graves disease	- measurement of RAIU, serum T_4 and T_3, RT_3U and FT_4I - thyroid enlargement - weakness, weight loss, nervous instability, tremor, intolerance to heat, hyperhidrosis, palpitation and hyperdefecation (thyroid function and heart rate are preferably used to monitor therapy)
Pemphigus Vulgaris	- oral or mucosal lesions (light microscopy examination of epidermis immunofluorescent detection of autoantibodies are preferred to monitor therapy)
Glomerulone-phritis	- hematuria - red cell cast excretion - proteinuria - fluid retention
scleroderma	- observation of afflicted tissue - esophageal dysfunction - rheumatoid factor - antinuclear and serum antinuclear antibodies - anticentromere antibodies - antibodies against specific scleroderma antigens
myositis	- observation of afflicted tissue - arthralgia - muscle weakness - muscle enzymes (creatin kinase, transaminase)
vasculitis	- observation of afflicted tissue (vascular and skin lesions)

Alternatively, patient improvement can be assessed by assay to determine whether there has been a significant reduction in the frequency of autoreactive T-cells; or in the frequency of autoreactive antibodies, or both.

"Oral" administration includes oral, enteral or intragastric administration, and more generally any administration of an active ingredient that brings the ingredient in contact with the immune system at the gut-associated lymphoid tissue.

"Parenteral" administration includes subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal or intrathecal administration. Intravenous administration is preferred. Parenteral administration must be free of co-

stimulatory substances which might cause an undesirable immune reaction.

Rationale

The present inventors conceived that it should be possible to suppress both Th1 and Th2 abnormal immune responses (whether these responses were directed against an external antigen or a self-antigen) via orally or parenterally induced tolerance.

The present inventors observed that maintenance of exposure to antigen appeared to be important for the persistence of tolerance in various different contexts: orally induced tolerance to OVA antigen in mice immunized with OVA (14); parenterally induced tolerance due to induction of clonal anergy (15); and anergic cells removed from contact with the antigen by transplantation in a mouse strain lacking the antigen (16). The inventors decided to test their hypothesis in the context of orally induced tolerance by extending the exposure of the immune system (by contact with the gut-associated lymphoid tissue) to orally administered antigen and attempting to define conditions for induction of tolerance against Th2 lymphocytes.

The simplest system for testing this was to make defined quantities of antigen available to the experimental animals throughout their daily activity period while being able to assess antigen consumption as well as its frequency. It was therefore decided to add the antigen used for immunization to the animals' drinking water.

Another consideration was to devise a system for testing the principle of this invention in a manner that would be of as general applicability as possible, in light of the present skill and knowledge in the art. It was decided to use OVA as the tolerizing agent (fed antigen) and as the immunogen. In Friedman, A. et al. 1994, PNAS (USA) 91:6688, it was demonstrated that Th1 and Th2 responses in the autoimmune disease model EAE (a model for multiple sclerosis) were similar to those seen in induction of immunity by OVA. Furthermore, oral tolerization had been effective in suppressing an immune

response to OVA (Richman, L.K., et al. J. Immunol. 121:2429, 1978). The applicability of this oral tolerization treatment (i.e. the treatment involving prolonged exposure to antigen through "multi-dose administration" as defined below) to various disease states characterized by one or more undesirable immune responses would depend on the ability of this regime to suppress various immune responses. The T-cells of the subject react to an antigen they recognize regardless of whether that antigen is endogenous (as in autoimmunity) or not (as in the present experimental models).

The present inventors found that multi-dose daily oral administration of antigen to a subject to be treated (i.e. a subject that mounts an immune response to that antigen) achieves the suppression of not only Th2 but also Th1 immune responses to the same antigen. "Multi-dose administration" or "multi-dose exposure" or "exposure to multiple doses" encompasses administration occurring at a plurality of spaced apart intervals during the same day as further described below, as well as continuous administration either by intragastric or parenteral infusion, or by ingestion of a sustained-release dosage form. The term thus refers to a schedule or frequency of administration.

The thus induced tolerance of not only Th2 by also Th1 lymphocyte responses entrained (and was confirmed by) profoundly suppressed numbers of *in vivo* secreted antigen-specific antibodies, including IgG1 and IgE (which are controlled by Th2 cytokines, with IgE being considered to be exclusively Th2-controlled) as well as IgG2a and IgG2b (which are controlled by IFN-γ, a Th1 cytokine).

Tolerance of both Th1 and Th2 responses to the multi-dose fed antigen was confirmed by: (i) failure of T-cells from animals fed antigen by multi-dose administration to proliferate *in vitro* to the fed antigen (which also had been used for immunization), as well as by (ii) suppression of Th1 and Th2 cytokine secretion and cytokine gene expression by the tolerized T-cells. In fact, suppression of cytokines was demonstrated by ELISA, CT.4S cell proliferation and RT-PCR

(reverse transcriptase polymerase chain reaction). These tests demonstrate tolerization of Th2 lymphocytes.

The present inventors were the first to achieve complete tolerization of Th2 lymphocytes as demonstrated both *in vitro* and *in vivo*.

Using another approach, the present inventors determined further that even larger amounts of antigen orally administered also bring about suppression of Th2 (and Th1) responses even when not administered in multiple daily doses. Specifically, in experiments involving transgenic mice expressing essentially only a T-cell receptor specific to OVA (Vα13/Vβ8.2TcR), considerable suppression of both Th2 and Th1 responses was achieved by feeding large amounts of antigen intermittently in single doses. The mechanism of suppression is substantially through deletion of antigen-specific Th2 and Th1 cells. The inventors concluded that this approach can be used to induce tolerance in Th2-mediated (antibody-mediated) responses associated with autoimmune disease. This "high-dose" oral tolerization (in which antigen can be administered only once daily or according to a less frequent administration schedule, as described below) can be used as an alternative or an adjunct to multi-dose oral tolerization. In fact, in a preferred embodiment, subjects to be treated will be administered both high doses of antigens and multiple daily doses.

In Examples 6-9, the animal model used involved animals that have only T-cells that are reactive with the same antigen as that used for immunization (OVA). The rationale for applicability of these findings to human antibody-mediated autoimmune disease is the same as that described above for the multi-dose experiments.

Amounts and Schedule (Frequency and Duration) for Oral Administration

Multi-dose daily autoantigen administration (and attendant delivery of relatively high amounts of antigen to a locus exposed to the treated subject's immune system) achieves suppression of Th2 as well as Th1 responses. (Suppression of

only Th1 requires only relatively modest amounts of antigen and intermittent administration: for example, in humans, rheumatoid arthritis symptoms have been suppressed with as little as 0.1 mg of collagen II administered once a day, for one month, followed by administration of 0.5 mg collagen II administered daily for two months.)

Relatively protracted exposure to antigen enhances tolerization of Th2 responses. For example, mice need to be fed with a multi-dose daily regimen requiring relatively large amounts of antigen (see infra) for more than 15 and preferably at least 20 days.

However, suppression of Th2 responses can also be achieved in mice by single dose intermittent (e.g., once daily, every other day or twice a week) administration of even higher doses of antigen. Again, continuing the administration over a period of time increases suppression.

Thus, the amount of antigen effective to suppress Th2 responses to that antigen depends partially on the schedule (frequency) of its administration. Suppression, even complete suppression of Th2 responses to a particular antigen (OVA) can be achieved by administering e.g. 4 mg of antigen in several divided daily doses to mice whereas the same amount (4 mg) of OVA, if administered once a day, achieved only a slight suppression of IL-2 (see, e.g. Table III).

A substantially higher amount of an antigen is needed to suppress Th2 responses if the antigen is administered once daily or intermittently. Suppression of Th2 responses is typically achieved after a number of feedings, in mice, typically three or more. Thus after three feedings of 5 or 50 mg of antigen on alternate days partial OVA-specific Th2 suppression (predominately T-cell deletion) was achieved in mice. Substantially more profound suppression resulted from feeding 500 mg of OVA according to the same schedule.

Accordingly, to induce suppression of both Th1 and Th2 responses in mice oral administration of daily amounts within the general range of 2 mg - 500 mg depending on the antigen would be useful, preferably, 5 mg to 100 mg. The schedule of administration should be at least 3 and preferably

at least 5 times a day, for smaller amounts within this range but may be less frequent e.g. once daily, once every other day, or twice a week as the amount of antigen per treatment is increased.

In humans, to suppress Th2 responses associated with antibody-mediated autoimmune disease using multi-dose oral administration, an autoantigen should be administered orally at least three and preferably at least five or six times a day at spaced apart intervals (e.g. with and/or between meals). For humans, the total daily dosage of multi-dose administration will be within the general range of 2.5 - 1,000 mgs of autoantigen depending on the autoantigen, preferably within the range of 15 - 500 mgs divided among several dosages (as stated above). No maximum effective number of dosages or total daily intake of antigen has been discerned. Twelve daily doses provide a practical limit.

When high-dose intermittent administration is used in humans, antigen could be administered less often: twice daily, once daily, three-times weekly, twice weekly or once a week. In that event, the amount of administered antigen should be within the range of 30 mg - 10 g per treatment depending on the antigen.

In either case, the duration of treatment in humans should be a minimum of two weeks, and typically three months, and may be continued indefinitely or as long as benefits persist. The treatment may be discontinued if desired (in the judgment of the attending physician) and the patient monitored for signs of relapse. If clinical symptoms or other disease indicators show that the patient is relapsing, treatment may resume.

As will be understood by those skilled in the art, the dosage will vary with the disease, the antigen administered and may vary with the sex, age, and physical condition of the patient as well as with other concurrent treatments being administered. Consequently, adjustment and refinement of one or both of the dosages used and the administration schedules will preferably be determined based on these factors and especially on the patient's response to the treatment. Such

determinations, however, require no more than routine experimentation, as illustrated in Examples A-C below.

Antigens that May be Used to Induce Tolerance

Suitable antigens include autoantigens (as defined in the present detailed description) specific for a particular antibody-mediated autoimmune disease.

Nonlimiting examples of autoantigens for each of various antibody-mediated autoimmune diseases are:

Disease	Antigen for Multi-dose Administration to Humans
Autoimmune Thyroiditis	thyrotropin receptor thyroglobulin thyroid stimulating hormone receptor (TSHR)
Systemic Lupus Erythematosus	nuclear antigens recognized by anti-nuclear antibodies, e.g., SS-A antigen; La antigen (also known as SS-P); DNA (both double stranded and single stranded); RNA; Sm-(Smith)antigen; nRNP (nuclear ribonucleoprotein) antigen; and fragments thereof such as the 60 kd and 52 kd proteins of SSA; b', d' and d proteins of Sm antigen; RNP 70 kd antigen; and RNPC of NRNP. These antigens have been described in Wagatsuma et al. Mol. Immunol. 30: 1491-1498, 1993; Priujn, in Manual of Biological Markers of Disease (BP4.2: 1-14) Kluwer Academic Publishers, Drodrechet/ Boston/London 1994; and Smeenk, (p. B2.1: 1-14); Ku antigen, see infra.
Myasthenia Gravis	acetylcholine receptor, Waser, et al., Eur. J. Pharm. 172: 231-238, 1989
Insulin Resistance	insulin receptor (e.g. U.S. Pat. No. 5,385,888)
Autoimmune Hemolytic Anemia	red blood cells and antigens associated with them, e.g. Rh antigens disclosed in Agre, P. et al. Blood, 78:551-563, 1991
Autoimmune Thrombocytopenic Purpura	platelets; platelet membrane antigens, e.g. glycoprotein IIb-IIIa, Kiefel, V. et al. Seminars in Hematology 29:26-33, 1992
Glomerulonephritis	glomerular basement membrane and antigens associated with it
Ulcerative Colitis	autoantigen (40kd) associated with disease, Das et al., J. Immunol. 139: 77-84, 1987; autoantigen target of perinuclear antineutrophil cytoplasm antibodies

Disease	Antigen for Multi-dose Administration to Humans
Pemphigus Vulgaris	desmoglein, keratins, Type XVII collagen, Type IV collagen
Primary Biliary Cirrhosis	Ku antigen (Factor E1BF) (EBP-80), Mimori et al., J. Clin. Invest. 68: 611-620 (1981); dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase
Myositis	aminoacyl-tRNA synthetases such as Jo-1 antigen PL-7, PL-12, EJ and OJ antigens; PM/ScI complex; PM/ScI-100; PM/ScI-75
Wegener's Granulomatosis	proteinase-3, Kao et al., J. Clin. Invest. 82: 1963-1973, 1988; antineutrophil cytoplasmic autoantibodies (ANCA)
Grave's disease	TSHR
Vasculitis	ANCA-antigens; serine protease proteinase-3
Scleroderma	DNA topoisomerase I (110KD); scleroderma 70 antigen (70KD); scleroderma 86 antigen (86KD); scleroderma 110 antigen; centromere proteins (CENP-A, CENP-B, CENP-C) Manual of Biol. Markers of Disease B.5. 2:1-17, 1994; PM/ScI antigen; PM/ScI-100; PM/ScI-75

All of the foregoing are well-known and extensively characterized in the literature. The nucleotide and amino acid sequences of several of the foregoing antigens have been elucidated and some are commercially available. Where individual antigens are not known, impure preparations containing lysates from afflicted tissue (from the same or a related species) can be used prepared by well-known techniques.

Additional autoantigens can be identified by screening antigens for binding with antibodies or activated T-cells from the patient.

Formulations

Administration of more than one autoantigen is possible, and in fact desirable when the patient's T-cells or autoreactive antibodies recognize more than one antigen. In cases where the autoantigen is unknown, entire tissue extracts

(i.e., tissue lysates) from the same or a related species can be administered.

Suitable formulations for use in tolerization of Th2-responses according to the present invention can be in any suitable orally administrable form. For example, a pill, a liquid, a capsule containing an effective amount of antigen. Each oral formulation may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents and salts as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures implying solid carriers well-known in the art. Capsules may be made from any pharmaceutically acceptable materials, such as gelatine or cellulose derivatives. Nonlimiting examples of solid carriers include starch, sugar, bentonite, silica and other commonly used inert ingredients. Diluents can include *inter alia* saline, syrup, dextrose and water.

The autoantigens used in the present invention can also be made up in liquid formulations or dosage forms such as, for example, suspensions or solutions in a physiologically acceptable aqueous liquid medium. Such liquid media include water, or suitable beverages, such as fruit juice or tea which will be convenient for the patient to sip at spaced apart intervals throughout the day. When given orally in liquid formulations the antigen may be dissolved or suspended in a physiologically acceptable liquid medium, and for this purpose the antigen may be solubilized by manipulation of its molecule (e.g., hydrolysis, partial hydrolysis or trypsinization) or adjustment of the pH within physiologically acceptable limits (e.g. 3.5 to 8). Alternatively, the antigen may be reduced to micronized form and suspended in a physiologically acceptable liquid medium. For parenteral administration the antigen should be administered in a solution.

Sustained released oral delivery systems are also contemplated and are preferred. Nonlimiting examples of sustained release oral dosage forms include those described in U.S. Patent No. 4,704,295 issued November 3, 1987; No. 4,556,552 issued December 3, 1985; No. 4,309,404 issued January

5, 1982; No. 4,309,406 issued January 5, 1982; No. 5,405,619 issued April 10, 1995; WO 85/02092 published May 23, 1985; No. 5,416,071 issued May 16, 1995; No. 5,371,109 issued December 6, 1994; No. 5,356,635 issued Oct. 18, 1994; No. 5,236,704 issued August 17, 1993; No. 5,151,272 issued September 29, 1992; No. 4,985,253 issued January 15, 1991; No. 4,895,724 issued January 23, 1990; No. 4,675,189 issued June 23, 1987.

Sustained release oral dosage forms coated with bioadhesives are preferred. Examples are compositions disclosed in EP 516,141; No. 4,226,848, Nagai et al., Oct. 1980; No. 4,713,243, Schiraldi et al., Dec. 1987; No. 4,940,587, Jenkins et al., July 1990; WO 85/02092; EPO 0 205 282; Smart, et al., J. Pharm. Pharmacol. 36:295-99, 1984; Sala et al., Proceed. Intern. Symp. Control. Rel. Bioact. Mater. 16:420-21, 1989; Hunter et al., International Journal of Pharmaceutics 17:59-64, 1983; Bioadhesion - Possibilities and Future Trends, Kellaway, Course No. 470, May 22-24, 1989.

Preferred commercially available sustained release formulations and devices (the latter can be used in infusion described below) include those marketed by ALZA Corporation, Palo Alto, CA, under tradenames ALZET, INFUSET, IVOS, OROS, OSMET, or described in one or more U.S. patents: No. 5,284,660 issued Feb. 9, 1994; No. 5,141,750 issued Aug. 25, 1992; No. 5,110,597 issued May 5, 1992; No. 4,917,895 issued April 17, 1990; No. 4,837,027 issued June 6, 1989; No. 3,993,073 issued Nov. 23, 1976; No. 3,948,262 issued April 6, 1976; No. 3,944,064 issued March 16, 1976; No. 3,699,963; PCT/US93/10077; PCT/US93/11660; EP 259013; and EP 354742.

Sustained release compositions and devices are particularly adapted for use in the present invention because they serve to prolong contact between the antigen and the gut-associated lymphoid tissue (GALT) and thus prolong contact between the antigen and the immune system. In addition, sustained release compositions obviate the need for discrete multi-dose administration of the antigen and permit the required amount of antigen to be delivered to GALT in one or two daily doses. This substantially improves patient compliance.

Parenteral Administration of Antigen -
An Alternative to Multi-Dose Oral Administration

An alternative method within the scope of the present invention for accomplishing tolerance of Th2 responses is a method comprising prolonged parenteral infusion of a subject to be treated with an autoantigen. For humans, the duration of each treatment will be, for example, from about 1 hour to about 24 hours of continuous infusion repeated at intervals of 1-4 weeks as needed. Upward or downward adjustments to the infusion time can be made based on the patient's response. A preferred route of parenteral administration is intravenous administration. Suitable parenterally deliverable amounts of antigen are within the range of about 2.5 to about 250 mg of antigen. Suitable formulations include sterile solutions of antigen suitable for parenteral administration in an appropriate medium (e.g., saline in distilled water etc.). Buffers, emulsifiers, salts and other optional ingredients suitable for such preparations can be included. Purified antigen should be administered without co-stimulatory factors (which would induce an immune response against the antigen). Suppression is accomplished by anergy or clonal deletion.

The invention is further described below by reference to examples, the purpose of which is to illustrate the present invention without limiting its scope.

All documents cited herein are incorporated by reference therein. In case of conflict, however, the present specification including its definitions will control.

Examples 1 - 5

Experimental Animals

Female BALB/C mice, 6-8 weeks of age, were used in all experiments. The mice were bred at the department of Animal Sciences, Hebrew University of Jerusalem, Rehovot, Israel, where they are periodically crossed with wild-type Balb/c mice. The animals were maintained in a temperature and light-controlled environment with free access to feed and water. During experiments mice fed with OVA intermittently several times during the day (see below) had free access to OVA

solution in water, instead of water alone. Each experimental group contained no less than 5 mice.

Antigens, Feedings and Immunizations

Antigens used were ovalbumin (OVA) (from Sigma Chemicals Co., St. Louis, MO) and purified protein derivative PPD (Statens, Denmark). Oral tolerance to OVA was induced by multi-dose oral exposure to a sterile solution of OVA in drinking water (1 mg/mL, unless otherwise stated) for a period of 20 days (unless otherwise stated). Each mouse consumed 4 ± 0.25 mL solution/day. In several experiments, mice were fed 20 intermittent boluses of OVA in water (4 mg OVA/feeding) over the period of 20 days. Mice were immunized against OVA by injecting 20 μg OVA/mouse, either emulsified 1:1 in CFA, or absorbed by 1 mg $(Al(OH)_3$. Injections (100 μl/mouse) were administered IP (for spleen and mesenteric lymph node [LN] analysis) or subcutaneously in hind foot-pads (for popliteal LN analysis).

Assay for anti-OVA antibodies

Presence of IgG1, IgG2a and IgG2b serum antibodies specific for OVA was tested by ELISA as described (4, 18). Serial two-fold dilutions of anti-OVA antisera were placed on ELISA plates (Nunc, Denmark), previously coated with OVA, followed by biotinylated rat anti-mouse IgG1, IgG2a or IgG2b monoclonal antibodies (Pharmingen, San Diego, CA), and finally by peroxidase-streptavidin (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, MD). Bound antibodies were detected by ABTS (KPL). Due to low serum concentrations of IgE, an OVA-specific IgE ELISA protocol was developed. Plates were coated with 2 μg/mL monoclonal anti-mouse IgE (Southern Biotechnology Associates, Birmingham, AL). After blocking, serial two-fold serum dilutions were added, followed by OVA (50 μg/mL in PBS), then by mouse anti-OVA hyper-immune serum (1:1000), and finally by peroxidase-goat anti-mouse IgG (γ chain specific) (KPL). Bound antibodies were detected by the addition of ABTS. Antibody titers are averages of no less than 5 individual mice and are expressed in absorbance units at 405 nm ± SEM.

Cell cultures

Spleen (erythrocyte depleted) and lymph node (LN) cell (popliteal and mesenteric) cultures were used for proliferation, cytokine secretion and cytokine gene expression assays. Cultures contained pooled cells from no less than 5 mice. The proliferation of T lymphocytes in spleen and LN cell cultures was assayed as described (14,19). Proliferation was measured by MTT oxidation (14,19), and results are averages of quadruplicate cultures expressed in absorbance units (at 570-630 nm) ± SEM. For cytokine secretion, 1 x 10^7 cells/well (in 1 mL) were cultured in 24 well plates (Nunc, Denmark) with or without OVA 1 mg/mL). Cytokine secretion was determined temporally in supernatants collected from these cultures and was ascertained to be optimal after 9 hours culture for IL-4 detection, 20 hours for IL-2 detection, and 48 hours for IFNγ detection. Collected supernatants were frozen and stored at -70°C till assayed (see below). For the assay of cytokine gene expression, 5 x 10^7 spleen cells/ml were cultured with or without OVA (1 mg/mL) for 6 hours. Following incubation, cells were collected and total RNA was isolated for RT-PCR (see below). DMEM was used for all cultures, and was supplemented with 100 U/ml penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (all supplied by Biological Industries, Beit Haemek, Israel), 5 x 10^{-5}M 2-mercaptoethanol and 0.5% syngeneic serum.

Cytokine assays

Levels of IL-4, IL-2 and IFNγ in supernatants were determined by capture ELISA as described (3,4,20). Briefly, supernatants were added to microtiter plates, previously coated with rat anti-mouse IL-4, IL-2 or IFNγ monoclonal antibodies (capture antibodies, Pharmingen) and blocked with BSA-diluent/blocking solution (KPL). Biotinylated rat anti-mouse IL-4, IL-2 or IFNγ monoclonal antibodies (detecting antibodies, Pharmingen) were added and followed by peroxidase-labeled streptavidin. Bound cytokine was detected by the addition of ABTS (Kirkegaard & Perry). Cytokine levels were calculated from a log-log plot of absorbance vs. concentration of

recombinant cytokines (Pharmingen), and results are expressed in pg/mL (for IL-4 and IL-2) or ng/mL (for IFNγ). Threshold sensitivities of ELISA assays were 5 pg/mL, 10 pg/mL and 2.5 ng/mL for IL-4, IL-2 and IFNγ, respectively. As a confirmation, IL-2 and IL-4 levels were also determined by bio-assay using the CTLL-2 (IL-2 dependent) and CT.4S (IL-4 dependent; kindly provided by Dr. W.E. Paul, NIH, Bethesda, MD) cell lines as described (4).

Analysis of cytokine mRNA levels

Total RNA was isolated from cultured spleen cells using a TRI reagent (Molecular Research Center, Inc., Cincinnati, OH), according to a protocol provided by the manufacturer. mRNA was then reverse transcribed into cDNA, and the expression levels of IL-4, IL-2, IFNγ and β-actin messages were determined by a quantitative polymerase chain reaction (PCR) using cytokine specific primers. β-actin, IL-2 and IFNγ primer sequences were from Stratagene (La Jolla, CA). IL-4 sequences were as follows: IL-4 sense: 5'-CAGCTAGTTGTCATCCTGCTC-3' (76-97) and IL-4 antisense: 5'-CAGGAAGTCTTTCAGTGATGTGAA-3' (445-421). All primers used spanned genomic introns such that any contaminating genomic DNA was detected by a higher molecular weight band. Quantitative PCR conditions were first established for all sets of primers using either cDNA from Con A activated spleen cells or plasmid DNA. These were subjected to 8 two-fold serial dilutions and amplified for 25 cycles at 95°C for 25 seconds, 60°C for 60 seconds and 72° for 60 seconds in a 9600 Geneamp PCR System (Perkin-Elmer Cetus Corp., Branchburg, NJ). [^{32}P]dCTP was added directly into the PCR reaction as described (21), and the products were resolved on 5.5% acrylamide gel. mRNA was quantitated using a β-scope (Intelligentics, Mountain View, CA). Tested cDNA samples were amplified undiluted and with two additional two-fold dilutions with cytokine specific primers, and at 1/100, 1/200 and 1/400 serial dilutions with β-actin primer sequences. The ratio of cytokine mRNA expression relative to β-actin was obtained for each dilution and expressed as the mean ratio ± SE (see all columns in Table III,

except for the columns labeled "ratio" which show the ratio of the value of the column labeled OVA over the value of the column labeled "medium" and thus compares the OVA readings against the background).

Statistical analysis

The statistical significance of differences between experimental groups was determined using unpaired two-tailed Student's t-test, with differences considered significant at $P < 0.05$.

Example 1: **Exposure of mice to multiple doses of OVA induces OVA-specific T lymphocyte unresponsiveness *in vitro*.**

To determine if tolerance could be induced in both Th1 and Th2 lymphocyte subsets, we exposed (orally tolerized) mice to OVA in their drinking water (ad libitum consumption). Exposure was continued for 20 days, and then mice were immunized by OVA-CFA. Control mice were primed by OVA-CFA or CFA alone. Spleen cell cultures were prepared 10 days after immunization and T lymphocyte proliferation in response to OVA (Fig. 1A) or PPD (Fig. 1B) was determined. T lymphocytes did not proliferate in response to OVA but exhibited a dose dependent response to PPD which was similar to that of the OVA-primed, non-tolerant control group (Fig. 1A-1D ; $P < 0.05$ for all antigen doses, representative of 6 experiments). Identical observations were made with LN (popliteal and mesenteric) cell cultures, with other protein antigens (human serum albumin [HSA], and hen egg lysozyme (HEL) and with $Al(OH)_3$ as adjuvant (data not shown). Thus, multi-dose oral exposure to external antigens in solution is not an immunogenic stimulus, but rather induces antigen-specific T lymphocyte unresponsiveness, as determined by absence of T lymphocyte proliferation.

In more detail, Figures 1A and 1B depict graphically the results of experiment involving multi-dose oral exposure to OVA and show that such exposure diminishes T lymphocyte proliferation response *in vitro*. Mice were multi-dose exposed to OVA in drinking water for 20 days and primed by OVA-CFA

(open triangles). Control mice were primed by OVA-CFA (filled circles) or CFA alone (open circles). Control mice were primed by OVA-CFA (filled circles) or CFA alone (open circles). Pooled erythrocyte-depleted spleen cells were prepared 10 days after immunization and stimulated by different concentrations of OVA (Fig. 1A) or PPD (Fig. 1B). Results are averages ± SEM of quadruplicate cultures containing pooled cells from at least 5 mice, and are expressed in absorbance units at 750-630 nm.

The results of this experiment show that multi-dose oral administration of antigens suppresses proliferation of antigen-specific T-lymphocytes and therefore suppresses a T-cell response.

Example 2: **Determination of absence of both Th1 and Th2 cytokine secretion in cultures derived from mice exposed to multiple doses of OVA in drinking water.**

Absence of proliferation is only partially indicative of tolerance, since activated non-dividing cells may produce cytokines (22,23). Thus, to further establish an *in vitro* state of tolerance in T lymphocytes derived from mice exposed to multiple doses of OVA in their drinking water, *in vitro* cytokine secretion was determined. Mice, multi-dose fed OVA (1 mg/mL) in drinking water for 20 days or non-fed controls, were immunized I.P. by OVA-CFA or OVA-$Al(OH)_3$ alone; two additional non-fed groups were primed by either CFA or $Al(OH)_3$ alone. Spleen cell cultures were prepared 10 days after immunization, incubated with or without OVA, and supernatants were collected to determine secretion of IL-4, IL-2 and IFNγ (Table 1, representative of 5 experiments).

In more detail, the results in Table 1 were generated as follows: pooled, erythrocyte-depleted, spleen cells (10 x 10^6/mL) were cultured 10 days after immunizations with or without OVA or PPD (1 mg/mL). Cytokine secretion was determined by ELISA. Cytokine levels were calculated from a log-log plot of absorbance vs concentration of recombinant cytokines. Results are averages of quadruplicate cultures pooled from at least 5 mice ± SEM. Values in bold lettering

indicate significant cytokine secretion above threshold levels ($P < 0.05$).

Mice exposed to multiple doses of OVA did not secrete IL-4, IL-2 or IFNγ in response to OVA (not at the designated time points and not at any other time point during a 48-hour culture period), suggesting that both Th2 (IL-4 secretors) and Th1 (IL-2 and IFNγ secretors) OVA specific lymphocytes were tolerant. This conclusion is supported by the following observations:

1) The three cytokines were secreted in response to OVA in control cultures derived from OVA-CFA and OVA-$Al(OH)_3$ primed mice, but not in those derived from mice primed by CFA or $Al(OH)_3$ alone, thus indicating OVA-specific activation of both Th1 and Th2 lymphocytes in two adjuvant systems ($P < 0.05$).

2) Tolerance was OVA-specific as evidenced by the capacity of cells from OVA-exposed mice to secrete cytokines in response to PPD ($P < 0.05$).

The possible presence of IL-4 and IL-2, undetected by ELISA, was further evaluated by bioassays using CT.4S (available from the NIH) and CTLL-2 (commercially available from the ATCC) cells, respectively; IL-2 and IL-4 were not detected in cultures derived from mice exposed to multiple doses of OVA, HSA or HEL, and identical observations were obtained with LN cell cultures (data not shown). The results of these experiments establish that multi-dose oral exposure to antigen in solution induces specific tolerance of Th2 and Th1 lymphocytes as determined by *in vitro* proliferation and cytokine secretion.

Example 3: **Determination that mice exposed to multi-doses of OVA in drinking water do not express Th1 and Th2 cytokine genes.**

To further evaluate the level of tolerance induced by exposing mice to multiple doses of OVA, we measured cytokine gene expression in response to OVA.

Mice, multi-dose fed or non-fed controls, were immunized by OVA-CFA; an additional non-fed group was primed

by CFA alone. Spleen cell cultures were prepared 10 days after immunization, incubated with or without OVA, and cells were collected to determine IL-4, IL-2 and IFNγ mRNA expression by quantitative PCR (Table 2, representative of 3 experiments).

In more detail, the results in Table 2 were generated as follows: mice were exposed to multiple doses of OVA in drinking water for 20 days (1 mg/mL OVA in water) and then immunized I.P. by OVA-CFA. Control non-fed mice were primed by OVA-CFA.

Pooled erythrocyte-depleted spleen cells (5×10^7/ml) were cultured 10 days after immunizations with or without OVA (1 mg/mL) for 6 hours. Cells were collected and total RNA was isolated. mRNA was reverse transcribed into cDNA and the expression levels of IL-4, IL-2, IFNγ and β-actin messages were determined by quantitative PCR using cytokine specific primers, as described in Materials and Methods. Visualized bands were quantitated using a β-scope, and cytokine mRNA expression values are relative to those of β-actin mRNA used as an internal control ± SEM. In addition, mRNA expression is presented as the ratio between mRNA expressed in response to OVA and that expressed in response to medium alone. Values in bold lettering indicate significant mRNA expression above levels expressed in response to medium alone ($P < 0.05$).

The data show that IL-4, IL-2 and IFNγ mRNA were not expressed in response to OVA by cells derived from mice multi-dose fed with OVA. In contrast, IL-4, IL-2 and IFNγ mRNAs were specifically elevated in control cultures ($P < 0.05$). Hence, the state of tolerance in Th2 and Th1 lymphocyte subsets was confirmed by absence of cytokine gene expression following a specific antigen stimulus. These results confirm that oral multi-dose administration of an antigen suppresses the occurrence of antigen-specific Th2 and Th1 cells and therefore suppresses an immune response to the antigen.

Example 4: **Determination that mice exposed to multiple doses OVA do not secrete Th2 and Th1 dependent antibodies *in vivo*.**

The previous observations indicated that multi-dose feeding of OVA induced a state of tolerance in Th2 and Th1 lymphocyte subsets, as determined by *in vitro* analysis. To confirm that a similar state was present *in vivo*, we studied profiles of antibodies produced in response to OVA stimulation in tolerant mice. Antibody isotype profiles serve as relative indicators of murine Th subset activity *in vivo*; IgG1 and IgE production is regulated by IL-4 and represents a Th2 mediated response, whereas IgG2a and IgG2b production is regulated by IFNγ and represents a Th1 mediated response (24,25). Mice were multi-dose fed with OVA for 20 days and then primed by OVA-CFA. Control mice were primed by OVA-CFA or CFA alone. Serum samples were collected temporally after immunization (15-60 days) and analyzed for OVA-specific IgG1, IgE, IgG2a and IgG2b secretion (Figs. 2A-2D, antibody titers in serum 15 days after immunization, representative of 5 experiments).

Figure 2 depicts graphically the results of experiments in which mice were multi-dose fed with OVA for 20 days (1 mg/ml) and primed by OVA-CFA (open triangles). Control mice were primed by OVA-CFA (filled circles) or by CFA alone (open circles). Serum samples were collected 15 days after immunization and individually assayed for IgG2a (Fig. 2A), IgG2b (Fig. 2B), IgG1 (Fig. 2C) and IgE (Fig. 2D) by ELISA. Antibody titers are averages ± SEM of titers from individual mice (for all isotypes) and are expressed in absorbance units at 405 nm. Each experimental group contained at least 5 mice.

As shown in Fig. 2, mice fed multiple doses of OVA did not produce any detectable antibody response to OVA; thus anti-OVA IgG1 and IgE, as well as IgG2a and IgG2b, levels were completely diminished. In contrast, control mice primed by OVA-CFA developed significant responses that consisted of all antibody subclasses, indicating that CFA was capable of supporting both Th2 and Th1 mediated responses *in vivo* ($Al[OH]_3$ promoted selective Th2 mediated antibody production and could not be used to confirm Th1 tolerance). Antibody production was intact in tolerant mice as evidenced by presence of all four

subclasses in response to PPD; levels were similar to those of control OVA-CFA immunized mice, and similar results were obtained 20, 30, 45 and 60 days after immunization (data not shown). Taken together, the results show that both Th2 and Th1 responses are susceptible to antigen-specific tolerance induction by multi-dose oral exposure to an amount of antigen above a threshold amount.

Example 5: **Characterization of the tolerogenic signal required for tolerization of Th2 lymphocytes.**

Since selective tolerization of Th1 lymphocytes was accomplished by means of a different feeding regimen (by a single or an intermittent feeding regimen; see e.g. ref. 4), it was of interest to determine comparative requirements for inducing Th2 lymphocyte tolerance. Three parameters were studied: 1) the rigidity of the feeding regimen, namely the necessity for multi-dose exposure compared with a less-frequent intermittent (e.g. once daily) feeding regimen; 2) the minimal antigen dosage required for effective tolerization of Th2 lymphocytes; 3) the minimal period required for effective tolerization of Th2 lymphocytes.

The importance of the feeding regimen for tolerization of Th2 lymphocytes was studied by comparing the degree of tolerance generated by multi-dose exposure to OVA in drinking water for 20 days to that generated by an intermittent (in this experiment on alternate days) feeding regimen in which mice received the same average dose of OVA (4 ± 0.25 mg/day). Mice were then immunized by OVA-CFA, and responses of both groups to OVA were compared 15 days after immunization (Table 3, representative of 4 experiments).

Table 3 was generated from data from the following experiment: mice were either exposed to multiple doses of OVA (1 mg/mL) or received daily boluses containing 4 mg/mL for 20 days.

Details of cytokine measurement are as in Table 1; concentrations are pg/mL for IL-2 and IL-4, and ng/mL for IFNγ. OVA specific responses are averages of quadruplicate cultures $\pm$ SEM, and values in bold lettering indicate significant

cytokine secretion above background threshold levels (see Table 1) ($P < 0.05$).

Serum IgG2a and IgE were measured 15 days after immunization by isotype specific ELISA, and anti-OVA specific responses are expressed in absorbance units at 405 nm. The values of IgG2a and IgE are from serum dilutions of 1:100 or 1:40 respectively, and are averages of at least 5 individual mice ± SEM. Values in boldface indicate significant secretion as compared to levels in naive serum (mean absorbance 0.15 ± 0.02) (P 0.05).

Th1 and Th2 tolerance resulted from the multi-dose feeding regimen (both *in vitro* cytokine production and *in vivo* antibody secretion), whereas the intermittent feeding regimen caused selective Th1 tolerance while Th2 responses (cytokine and antibody) were unchanged, apart from a minor reduction in IL-4 secretion ($P < 0.05$). Hence, the method of antigen exposure (multi-dose daily vs. intermittent) at the particular level of antigen administered was important for generation of Th2 tolerance.

The minimal antigen dosage required for effective tolerization of Th2 lymphocytes was determined by multi-dose exposure of mice to different concentrations of OVA in drinking water (0-1 mg/mL) for 20 days. OVA specific cytokine production *in vitro* and anti-OVA IgG1 and IgG2a production were assayed 15 days after immunizing mice with OVA-CFA (Fig. 3, representative of 4 experiments).

In the experiment that gave rise to Fig. 3, mice were multi-dose fed with different dosages of OVA for 20 days, and primed by OVA-CFA. Spleens and serum samples were collected 15 days after immunization to determine cytokine (circles) and antibody (triangles) secretion respectively. Erythrocyte-depleted spleen cells were cultured as detailed in the description of the experiment for Table 1, and supernatants were added to cultures of CTLL-2 or CT.4S cells for detection of IL-2 (solid circles) and IL-4 (open circles) respectively. Results are averages ± SEM of quadruplicate cultures and are expressed in absorbance units at 570-630 nm. IgG2a (solid triangles) and IgG1 (open triangles) were determined by ELISA.

Antibody titers are averages of antibody binding ± SEM from individual mice at a serum dilution of 1:400 and are expressed in absorbance units at 405nm. Each experimental group contained at least 5 mice.

Results in Fig. 3 show that tolerization of cytokine secretion *in vitro* and antibody responses *in vivo* required multi-dose exposure to 1 mg/mL OVA ($P < 0.05$). A dosage of 0.01 mg/mL had no effect on T lymphocyte functions ($P > 0.05$), and 0.1 mg/mL had only marginal effects on the measured functions ($P = 0.05$). Hence, effective oral tolerization of both Th1 and Th2 controlled responses (cytokine and antibody production) required dosages of this antigen in excess of 0.1 mg/mL. This shows that there is a minimum effective amount of orally administered antigen.

The minimal period required for effective tolerization of Th2 lymphocytes was determined by evaluating the temporal degree of tolerance generated *in vivo* by exposing mice to multiple doses of OVA (1 mg/mL). Mice were immunized temporally after feeding was initiated, and serum samples were collected, in each case, 15 days after immunization, and then assayed by ELISA for IgG1 and IgG2a (Fig. 4, representative of 5 experiments).

For the experiment of Fig. 4, mice were exposed to multiple doses of OVA for different periods (1-20 days). After each exposure period mice were immunized by OVA-CFA. Individual sera were prepared 15 days later and assayed by ELISA for anti-OVA IgG1 (open circles) and IgG2a (solid circles). Results are average antibody binding ± SEM from individual mice at a serum dilution of 1:400, and are presented in absorbance units at 405nm. Each experimental group contained at least 5 mice. Similar binding patterns were seen at serum dilutions ranging from 1:10 up to 1:800; antibody activity attained background values (0.155) at a dilution of 1:1600.

As shown in Fig. 4, Th1 mediated responses were most sensitive to tolerance induction: a single-day period of multi-dose exposure to OVA was sufficient to dramatically reduce specific IgG2a production (Th1-controlled), which essentially

ceased after 5 days of exposure. In comparison, Th2 controlled responses were highly resistant to tolerance induction and became so only after 20 days of multi-dose exposure to antigen: anti-OVA IgG1 production gradually diminished with time of exposure to OVA and attained background values only after 15-20 days of exposure. To summarize, oral tolerization of Th1 lymphocytes was achieved after a brief and intermittent exposure period to antigen. On the other hand, oral tolerization of Th2 lymphocytes was achieved only after extended periods of exposure to antigen and, at this amount per feeding, antigen needed to be administered in a multi-dose daily schedule. This shows that there is a minimum effective period of exposure to antigen, which is longer for Th2 than for Th1 suppression.

The results of this experiment show that there is a minimum effective amount of an antigen for inducing tolerance of a Th2 response specific to that antigen. The results also show that there is an interrelationship between the antigen amount fed and the frequency of the administration on one hand, and the effectiveness of the suppression on the other. More frequent (multi-dose daily) administration increases the effectiveness of tolerization. Moreover, oral tolerization of Th2 responses requires a longer period of treatment than oral tolerization of Th1 responses.

Example 6: **Frequency of CD4+, Vβ8.2+ T cells in Peyer's patch following antigen feeding.**

Ovalbumin (OVA) specific TcR-transgenic mice (n=6-9/group) that express the Vα13/Vβ8.2 TcR on 97% of peripheral T cells (Murphy et al., *Science* **250**, 1720-23 (1990)) were fed every other day for a total of up to five feedings with 0.5, 5, or 500 mg OVA. Members from each group were sacrificed either prior to the initiation of feeding or 24 hr after each feeding to provide data for no feeding of for intermediate feeding frequencies. Peyer's patches (5-8 per mouse) were harvested from the small intestine and a single cell suspension was prepared as described in Santos et al. *Cell. Immunol.* **157**, 439-447 (1994). Cells were first centrifuged through a ficoll-

isopaque gradient and then stained for CD4 (with PE-conjugated YTS 191.1 mAb, Caltag, San Francisco, CA) and Vβ8.2 (with FITC-conjugated MR5-2 mAb, Pharmingen, San Diego, CA). Fluorescence was analyzed on a Becton-Dickinson FACSort using Lysis II software. Data collection was gated on live cells through propidium iodide exclusion and data represent 10,000 events presented as probability (15%) contours. The percentage of Vβ8.2+ T-cells which are CD4+ as seen with each additional feeding is graphed in Figure 5A. Each data point in the Figure represents an average from 6-10 mice pooled from three independent experiments. The statistical significance (chi square analysis) of frequency differences of CD4+, Vβ8.2+ T cells among the various groups was as follows: for 0.5 mg fed, $p < 0.001$ versus 5 mg, 500 mg and unfed after 3 and 5 feedings; for 5 mg and 500 mg fed, $p < 0.001$ versus unfed after 3 and 5 feedings.

Exposure to small doses of OVA steadily increases the percentage of Vβ8.2+ cells that are CD4+ in the Peyer's patches, while larger doses -- 5 mg, 50 mg (not shown but similar to 5 mg) and 500 mg -- decrease the number which are CD4+. As seen in Figure 5A, in animals fed 500 mg, by the third feeding the percentage of CD4+, Vβ8.2+ T cells has decreased from 20% (unfed) to less than 1.5%. The decrease was not the result of an increase in the non-T cell population, as feeding was also associated with a 10-25% decrease in the total number of Peyer's patch cells. No effect was observed when OVA-TcR transgenic animals were fed 500 mg of bovine serum albumin and feeding 500 mg OVA did not affect the T cell frequency in Peyer's patches of non-transgenic animals (data not shown).

With the 500 mg feeding, a transient increase in CD4+ cell population was seen after one feeding. This was further investigated in Example 8.

There was no infiltration by CD4+,TcRVβ8.2+T cells of other lymphoid or nonlymphoid organs such as lung or liver (based on animals feed 500 mg OVA 3 - 5 times).

These results demonstrate that antigen-specific CD4+ T-cells are considerably suppressed following feeding of a

large amount of antigen for several times though neither multi-dose nor daily feedings are required at this level of antigen per feeding. These results complement the daily multi-dose experiments described above in showing high suppression antigen-specific T-cells with several, intermittent, frequent (though not necessarily daily) feedings of larger doses of antigen.

Figures 5B and 5C are fluorescence (FACS) contour plots of Peyer's patches from animals fed 5 times with either PBS (Fig. 5B) or OVA (Fig. 5C). These panels show the same data described above but illustrate pictorially the virtual absence of CD4+ cells from the upper right-hand quadrant of the FACS plot.

Example 7: **Frequency of apoptosis of Vβ8.2+ T cells in Peyer's patch following antigen feeding.**

To determine whether the loss of CD4+,Vβ8.2+ cells occurred by depletion, the following experiment was performed:

Peyer's patch cells as prepared in Example 6 were stained for Vβ8.2 with FITC-conjugated MR5-2 mAb and for degraded DNA with acridine orange (Sigma, St. Louis, MO). Fluorescence was analyzed as in Example 6, and data collection was gated on live Vβ8.2+ cells. Data represent 10,000 events presented as probability (15%) contours. To demonstrate apoptosis, cells were stained with acridine orange using a modified method of Hardin et al. (*J. Immunol. Meth.* **154**, 99-107 (1992)). Briefly, 10^6 ficoll separated cells were first stained for Vβ8.2 and then incubated in 100 μl of DMEM culture medium containing 10 μg of acridine orange at 25°C for 15 min. Cells were then washed and examined directly without fixation. The number of Vβ8.2+ apoptotic cells versus feeding over time is graphed in Figure 6A. Each data point in Figure 6A represents an average from 6-10 mice pooled from three independent experiments. The statistical significance (chi square analysis) of the frequency differences of apoptotic Vβ8.2+ T cells was as follows: for 500 mg fed, $p < 0.001$ after 2 feedings versus 5 mg., 0.5 mg, and unfed; for 5 mg $p < 0.01$ versus 0.5 mg and unfed after 3 and 5 feedings.

The Figure 6A graph indicates that feeding of 500 mg of OVA sharply increases the number of apoptotic Vβ8.2+ T-cells after the second feeding, while 5 mg induces only a gradual apoptosis increase (to 8% by the 5th feeding), and 0.5 mg induces minimal apoptosis. The increase of apoptotic cells seen in animals fed 500 mg returned to background levels by the 3rd feeding. In the animals fed 5 mg, the progressive increase reached 8% by the 5th feeding, whereas there was consistently only a minimal number of cells undergoing apoptosis in animals fed 0.5 mg. Furthermore, there was no increased deletion of CD4+, Vβ8.2+ cells observed in animals fed 0.5 mg twenty times over a one-month period (data not shown).

In situ labeling of cells for degraded DNA demonstrated a large percentage (up to 10%) of cells undergoing programmed cell death in the dome area of Peyer's patches from mice fed 500 mg OVA twice. FALS plots Fig. 6B and 6C illustrate this. This was not observed in control animals (fed 1 mg HEL) and was only minimally observed in animals fed 1 mg OVA (data not shown).

These results show that feeding large amounts of antigen induces clonal deletion of antigen-specific T-cells, which increases with repeated intermittent feeding.

Example 8: **Quantification of T cell activation in Peyer's patch following antigen feeding.**

The following experiment shows that T-cell deletion after high doses of oral antigen is preceded by T-cell activation.

Peyer's patch cells as prepared in Example 6 were stained for Vβ8.2 with FITC-conjugated MR5-2 mAb and for total DNA with propidium iodide (Noguchi in *Current Protocols in Immunology*, ed. Coligan et al., Wiley & Sons, Secaucus, N.J., 1994). Stimulation is measured by determining the number of Vβ8.2+ cells with high DNA content through propidium iodide staining because such cells are actively dividing, that is, they are in the S/G2-M phase of the cell cycle. Fluorescence was analyzed as in Example 7. The number of Vβ8.2+ cells in S/G2-M phase versus feeding over time is graphically

represented in Figure 7 with each data point representing a pooled average from 6-10 mice in three separate experiments. The statistical significance (chi square analysis) of the frequency differences of S/G2-M, Vβ8.2+ T cells was as follows: for 500 mg fed, $p < 0.001$ after 1-3 feedings versus unfed; for 0.5 and 5 mg fed, $p < 0.01$ versus unfed at all time points.

The Fig. 7A graph shows that feeding of 500 mg of OVA induces an initial stimulation of Vβ8.2+ cells which rapidly declines, presumably due to cell death, while stimulation induced by 5 mg and 0.5 mg feedings rise more slowly and tend to plateau at 4-5 feedings. Specifically, after one feeding of 500 mg OVA the percentage of activated cells rose to 7% and returned to 0 after five feedings. An increase in the percentage of activated cells also occurred in animals fed 0.5 and 5 mg and was maximal after 3 feedings although deletion was only observed in 5 mg fed animals. In addition, approximately 18% of T cells in animals fed two times with 500 mg OVA express the cell surface activation marker CD44 with low or no CD45RB, demonstrating T cell activation following oral antigen and that this activation precedes deletion (data not shown). Figs. 7B and 7C are FALS plots v. DNA content comparing unfed animals with animals fed 5 mg OVA once.

These results show that large amounts of antigen administered orally on an intermittent schedule of frequent but not necessarily daily feedings cause activation-induced apoptosis in antigen-specific T-cells. Indeed, in this and experiments 6, 7 and 9, considerable antigen-specific induced hyporesponsiveness of T-cells was observed even though the total number of OVA-specific T-cells is very high in the transgenic animal model used.

Example 9: **Measurement of activation and tolerization of Th1 and Th2 cells**

To determine the subtype of the T-cells being deleted, the following experiment was performed:

OVA-TcR transgenic mice were fed and sacrificed as described in Example 6. Splenocytes, 4×10^5 cells/well, were cultured in 0.2 ml of serum-free medium containing various

concentrations of OVA. Peyer's patches were not used because they contained an inadequate number of cells for multiple cytokine assays. Culture supernatants were collected after 40 hrs (for IL-2, IL-4, IL-10, IFN-γ) or 72 hrs (for TCF-β). Cytokine concentration was determined by ELISA. Quantitative ELISA for IL-2, IL-4, IL-10 and IFN-γ was performed using paired mAbs specific for corresponding cytokines per manufacturer's recommendations (Pharmingen, San Diego, CA). Active TGF-β1 (without acid treatment) was determined by a sandwich ELISA as described in Friedman & Weiner, *Proc. Natl. Acad. Sci. USA* **91**, 6688-6692 (1994). The results are in Figs. 8A - 8E.

Data presented represent mean of cultures with 1 mg/ml of OVA minus mean of cultures with 1 mg/ml of HEL. Each data point represents an average from 4-6 mice; the standard deviation is within 15% of the mean. The experiment was repeated 3 times with similar results. The statistical significance (as determined by Student's t test) for OVA fed versus unfed was as follows: $p < 0.0001$ for IL-2 after feeding 500 mg OVA 2-5 times; $p < 0.001$ for IL-4 and IL-10 after feeding 5 mg or 500 mg OVA 3 times; $p < 0.001$ for IL-4 and IL-10 after feeding 0.5 mg OVA 5 times. $P < 0.001$ for IFN-γ after feeding 5 mg or 500 mg OVA 3 times; $p < 0.0001$ for TGF-β after feeding OVA 3-5 times at all doses.

As seen in Figure 8, feeding of 500 mg (and 5 mg) of OVA initially enhances both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines in the spleen which is completely lost with continued feeding, while IL-2 secretion decreases without prior enhancement. Feeding of 0.5 mg progressively enhances IL-4 and IL-10, with a minimal effect on the production of IL-2 and IFN-γ. (These cytokine changes are consistent with the increase of CD4+,Vβ8.2+ cells following low-dose feeding in Example 6 and the decrease of these cells in Example 7 following high-dose feeding.) This indicates that a minimum dose of antigen is required for suppression of a cytokine profile, and that this amount is higher for suppression of the Th2 cytokine profile.

All three feeding regimens enhanced the production of TGF-β by OVA-specific T-cells. This indicates that antigen-specific T-cells secreting the nonspecific immunosuppressive Factor TGF-β are resistant to deletion by this tolerization regime. However, this is beneficial to treatment of autoimmune disease because TGF-β has the property of suppressing all immune responses in the vicinity of its release, including autoimmune responses.

Further Experiments

Splenic T cell proliferative responses were greater than 90% suppressed in OVA fed vs. nonfed animals (3,432$\pm$ 52 vs. 47,079$\pm$6,131 ΔCPM) measured by tritiated thymidine uptake and antibody responses (measured by ELISA) were suppressed by 50-75% in mice fed 500 mg OVA 5 times followed by subcutaneous immunization with 100 μg OVA/CFA. Anti-OVA IgM titers measured by ELISA were reduced from 512$\pm$43 in non-fed to 128$\pm$21 in the fed mice; IgG1 was reduced from 32$\pm$5 to 16$\pm$7; and IgG2a from 256$\pm$14 to 128$\pm$36. There were few or no detectable anti-OVA antibodies in naive transgenic mice, which indicates that all the anti-OVA antibodies were induced by immunization with OVA. In addition to deletion, evidence of anergy was also observed in mice fed 500 mg OVA. Specifically, the reduced splenic T cell proliferative responses could be partially reversed (from 3,432$\pm$52 to 24,227$\pm$1468 ΔCPM) by preculture of cells with recombinant IL-2 indicating that a number of antigen-specific T-cells became unresponsive not due to apoptosis, but due to anergy.

The foregoing experiments confirm both Th1 and Th2 suppression can be achieved using intermittent oral administration of large amounts of antigen. Thus, when treating antibody-mediated autoimmune diseases, an oral feeding regimen involving large amounts of autoantigen administered in single doses intermittently is indicated as one effective way of suppressing Th2 responses to that antigen.

Examples of Human Treatment

Example A: *Myasthenia Gravis*

An individual afflicted with *myasthenia gravis* is first orally administered 2.5 mg. of nicotinic acetylcholine receptor once a day for 1 week to eliminate the unlikely possibility of adverse reaction. Following this, the daily dosage is increased to 10 mg for a period of one to two weeks at the end of which the patient's antibody responses are measured using immunoassay (for reactivity to acetylcholine receptor). If no improvement is seen the daily dosage is increased (progressively) to 25, 50 or 100 mg etc. (and the antibody responses are monitored weekly or every two weeks) until an effective dosage is determined. (The number of daily doses may also be increased to six daily either instead of or in addition to an increase in total daily amount of antigen administered.) Once an effective amount and administration schedule has been identified for this patient (over the course of no more than several weeks) therapy continues at this amount and schedule for at least three months. Periodically, muscle strength is tested to monitor progress. (Muscle strength can also be used as an indicator of treatment efficacy instead of antibody responses during the phase of determining appropriate dosage and schedule.)

Example B: **Autoimmune Thrombocytopenic Purpura**

An individual afflicted with this disease is subjected to the same regimen as in Example A except that platelet count is monitored (weekly) and preferably glycoprotein IIb-IIIa are used as the orally administered autoantigen. A dosage is effective when increased platelet counts are normal or approach normal but do not increase further with additional orally administered antigen. Alternatively, an immunoassay measuring antibodies to platelets can be used to monitor the patient's progress.

Example C: **Autoimmune Thyroiditis**

An individual suffering from Hashimoto's disease is subjected to the same regimen as above but thyroglobulin is used as the antigen at the same amounts as in Example A.

Effectiveness of a particular regimen, is assessed and the patient's progress is monitored by decrease in antithyro-globulin autoreactive antibody. Preferably, the patient should receive treatment at a particular dose for at least 3 - 4 weeks before effectiveness can be assessed. If indicated, the number of daily dosages can be increased.

The same procedure can be followed for a patient suffering from Grave's disease, another type of autoimmune thyroiditis except that TSHR would be the autoantigen orally administered and therapeutic progress will be monitored, e.g., by decrease in anti-TSHR autoreactive antibody titer, or TSH levels.

The invention has been described above by reference to preferred embodiments. It will be understood that many modifications are possible within the scope of the claims that follow.

All documents, patents and patent applications are incorporated by reference in their entirety. In case of conflict, the present disclosure including its definitions controls.

Table 1.

Continuous exposure to OVA diminishes cytokine secretion *in vitro*.

OVA Fed	Immunization	IL-4 (9 h, pg/ml)		IL-2 (20 h, pg/ml)		IFNγ (48 h, ng/ml)	
		OVA	PPD	OVA	PPD	OVA	PPD
--	CFA	< 5	94±7	< 10	250±13	< 2.9	75±4
--	OVA-CFA	41±3	87±5	133±7	235±17	37±4	87±9
Continuous	OVA-CFA	< 5	98±8	< 10	262±19	< 2.8	75±7
--	$Al(OH)_3$	< 5	ND	< 10	ND	< 2.6	ND
--	OVA-$Al(OH)_3$	32±4	ND	55±2	ND	21±2	ND
Continuous	OVA-$Al(OH)_3$	< 5	ND	<10	ND	< 2.9	ND

Table 2.

Effect of continuous exposure to OVA on cytokine mRNA expression

OVA	1L-4 mRNA			IL-2 mRNA			IFNγ mRNA		
Fed	Medium	OVA	Ratio	Medium	OVA	Ratio	Medium	OVA	Ratio
--	0.7±0.1	2.1±0.1	3.0	2.9+1.0	5.8±1.0	2.0	0.9±0.2	5.9±0.5	6.6
Continuous	1.0±0.0	1.0±0.0	1.0	0.3±0.0	0.2±0.0	0.7	4.2±0.6	2.4+0.2	0.6

Table 3.

Effect of feeding regimen on OVA specific tolerization of Th1 and Th2 lymphocytes

OVA Fed	Immunization	Cytokine Secretion			Antibody Secretion	
		IL-4	IL-2	IFNγ	IgE	IgG2a
--	CFA	< 5	< 10	3.2±0.3	0.13±0.04	0.15±0.03
--	OVA-CFA	35.4±1.8	99.8±7.4	43.5±4.5	0.65±0.06	0.95±0.07
Intermittent	OVA-CFA	24.8±0.9	< 10	2.8±0.3	0.60±0.05	0.19±0.01
Continuous	OVA-CFA	< 5	< 10	3.1±0.2	0.18±0.03	0.17±0.04

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WE CLAIM:

1. A method for treating a mammal suffering from an antibody-mediated autoimmune disease comprising

orally administering to said mammal at least one autoantigen specific for said disease at a predetermined frequency of administration; and

continuing said administration at said frequency at least until at least one clinical symptom of said disease has been reduced or until at least one clinical indicator associated with said disease has attained or approached its normal value.

2. The method of claim 1 wherein the amount of said antigen, the frequency of said administration, and said period of time are effective to suppress a Th2 cell-mediated autoimmune response associated with said disease.

3. The method of claim 2 wherein said mammal is a human.

4. The method of claim 3 wherein said disease is selected from the group consisting of: systemic lupus erythematosus, autoimmune thyroiditis, myasthenia gravis, glomerulonephritis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, pemphigus vulgaris, Grave's disease, Type II-diabetes, insulin resistance, and pernicious anemia.

5. The method of claim 3 wherein said frequency of administration comprises administering said amount of said autoantigen at multiple doses at spaced apart intervals during a single day.

6. The method of claim 5 which comprises administering at least 6 doses of said autoantigen per day.

7. The method of claim 3 comprising continuing said administration for a period of time of at least two weeks.

8. The method of claim 5 wherein said amount is within the range of 5 to 1000 mg of said autoantigen per day.

9. The method of claim 1 comprising administering to said mammal a sustained release oral dosage form comprising said autoantigen and formulated to release said autoantigen in the gastrointestinal tract of said mammal for a period of time within the range of between about 6 and between about 12 hours.

10. A method for treating a mammal suffering from an antibody-mediated autoimmune disease, which comprises orally administering to said mammal an autoantigen specific for said disease and continuing said administration for a period of time sufficient to accomplish at least one of the following:

reduce the number of autoreactive Th2 cells in said mammal recognizing said autoantigen;

reduce the number of autoreactive antibodies in said mammal recognizing said autoantigen; and

eliminate or alleviate in said mammal at least one clinical symptom or indicator associated with said disease.

11. A method for suppressing an autoimmune response in a mammal in need of treatment, said mammal having at least one of autoreactive Th2 cells and autoreactive antibodies that recognize an autoantigen, the occurrence of said Th2 cells and said antibodies being associated with an antibody-mediated autoimmune disease, the method comprising orally administering to said mammal said autoantigen in an amount, at a frequency of administration, and for a period of time sufficient to accomplish at least one of the following:

decrease the number of said Th2 cells in said mammal;

decrease the number of said autoreactive antibodies in said mammal; and

eliminate or alleviate for said mammal at least one clinical symptom or indicator associated with said disease.

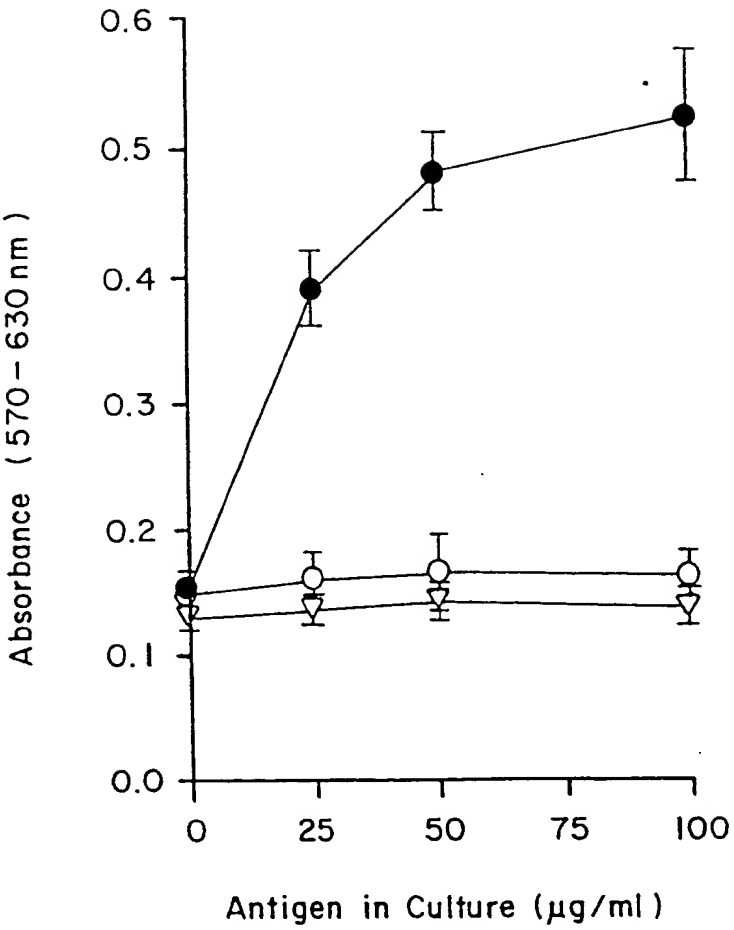
12. The method of claim 11, wherein said frequency comprises administering multiple doses of said autoantigen daily at spaced apart intervals within each day.

13. A method for treating a mammal suffering from an antibody-mediated autoimmune disease comprising

parenterally administering to said mammal at least one autoantigen specific for said disease;and

continuing said administration for a period of time; the amount of said antigen and said period of time being effective to suppress a Th2 cell mediated autoimmune response associated with said disease.

FIG. 1A


0.6
0.5
0.4
0.3
0.2
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0.0
Absorbance (570–630 nm)
0
25
50
75
100
Antigen in Culture (µg/ml)

FIG. 1B

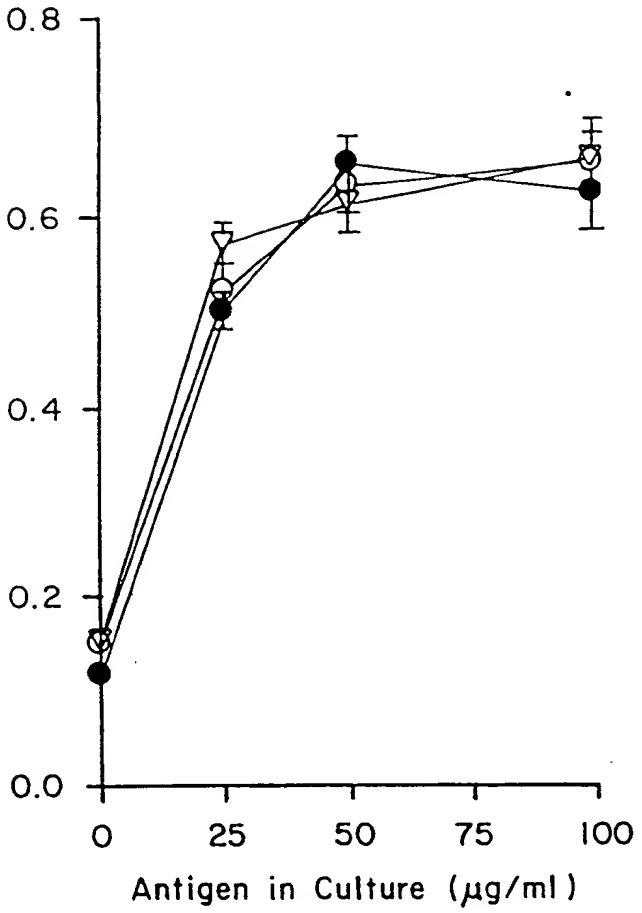
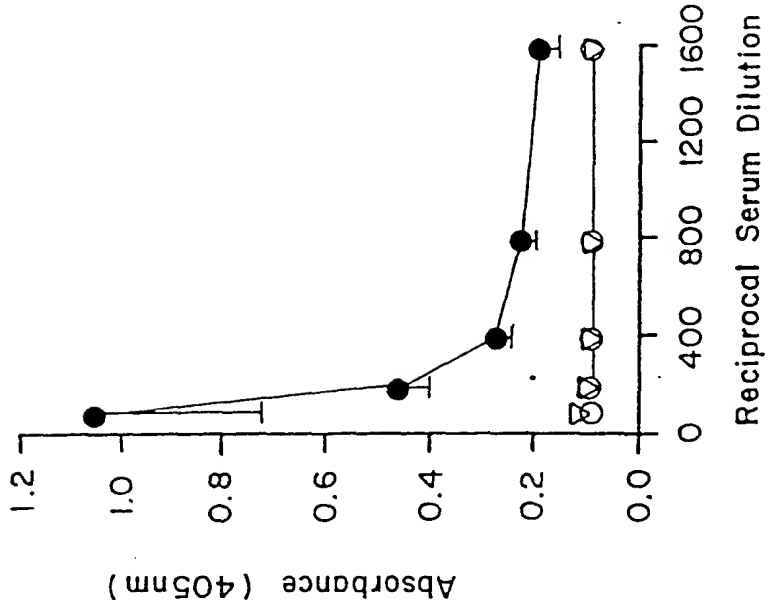


FIG. 2B


Absorbance (405nm)
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1.0
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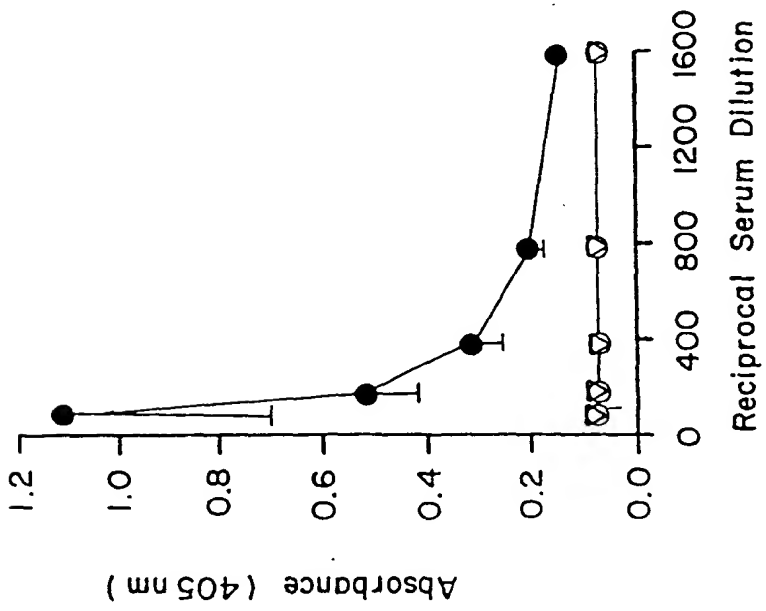
0.4
0.2
0.0
0
400
800
1200
1600
Reciprocal Serum Dilution

FIG. 2A


Absorbance (405 nm)
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0.2
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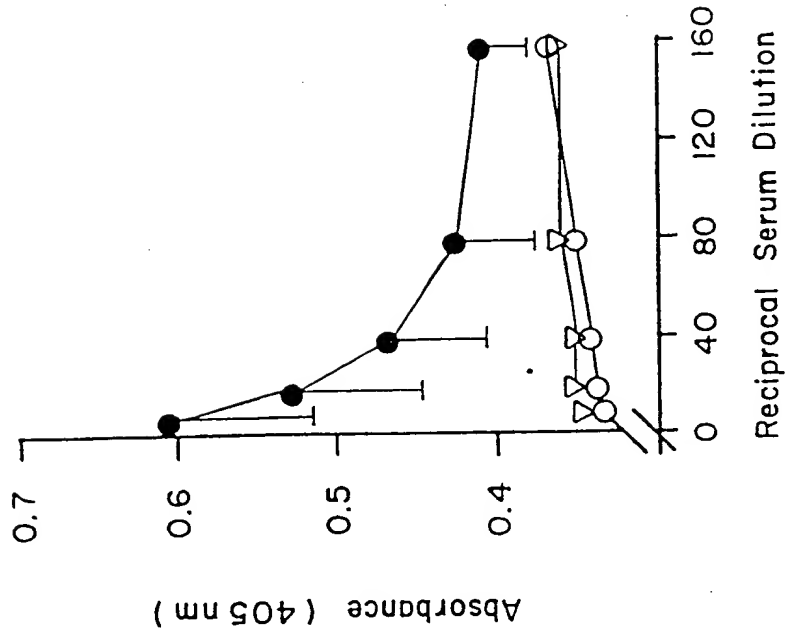
0
400
800
1200
1600
Reciprocal Serum Dilution

FIG. 2D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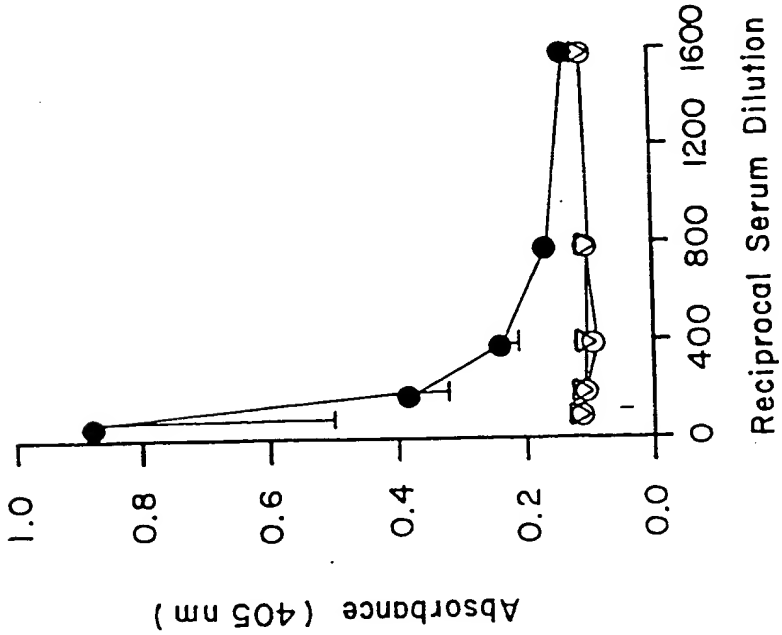
40
80
120
160
Reciprocal Serum Dilution

FIG. 2C


Absorbance (405 nm)
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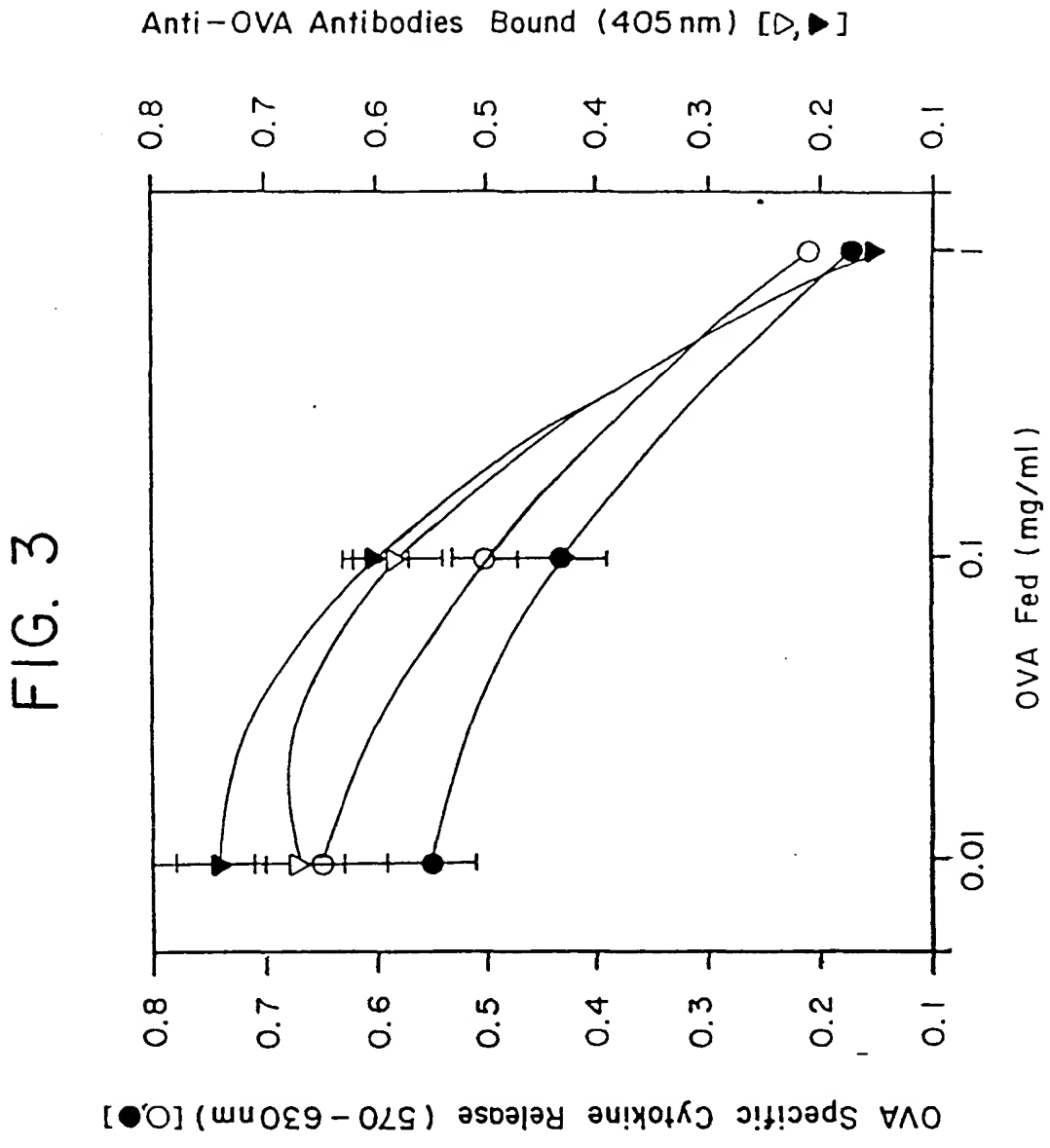
0.4
0.2
0.0
0
400
800
1200
1600
Reciprocal Serum Dilution

FIG. 3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0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OVA Fed (mg/ml)
1
0.1
0.01
OVA Specific Cytokine Release (570-630nm) [○,●]

FIG. 4

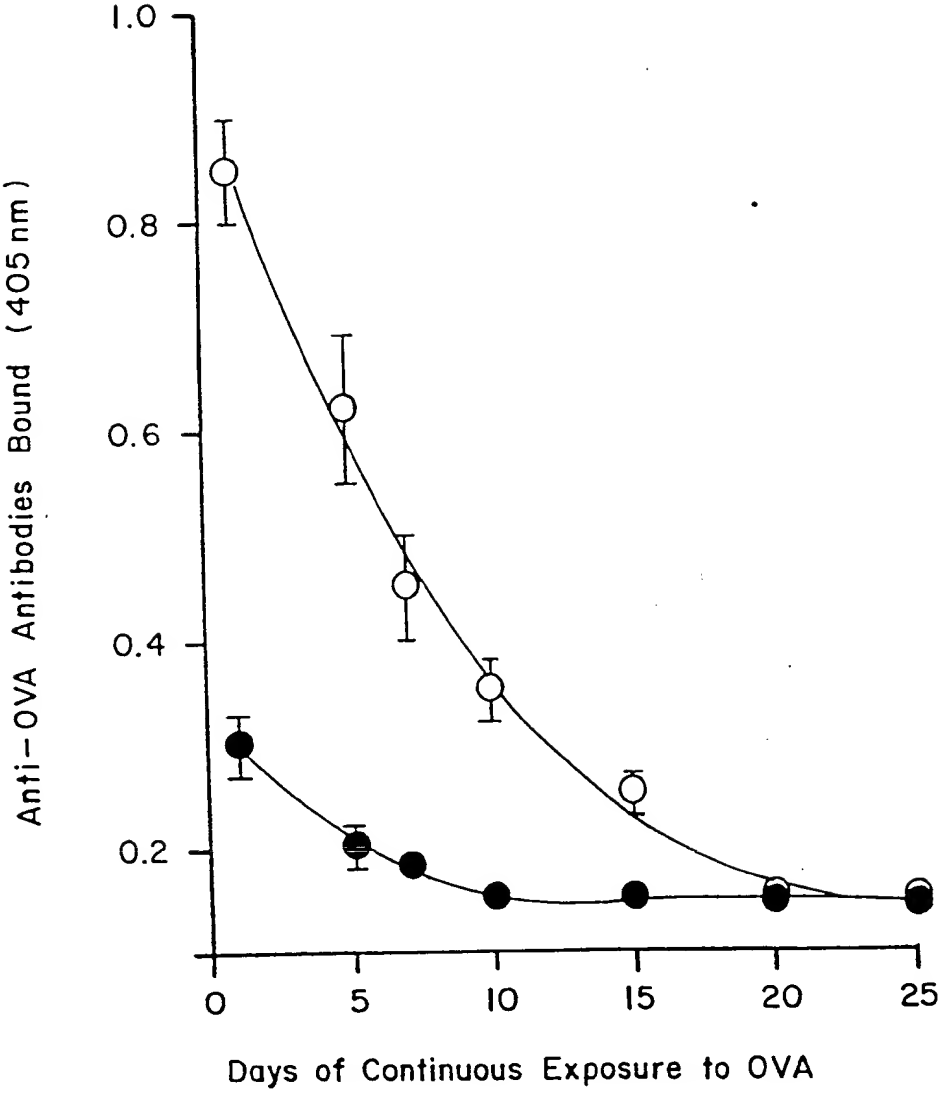
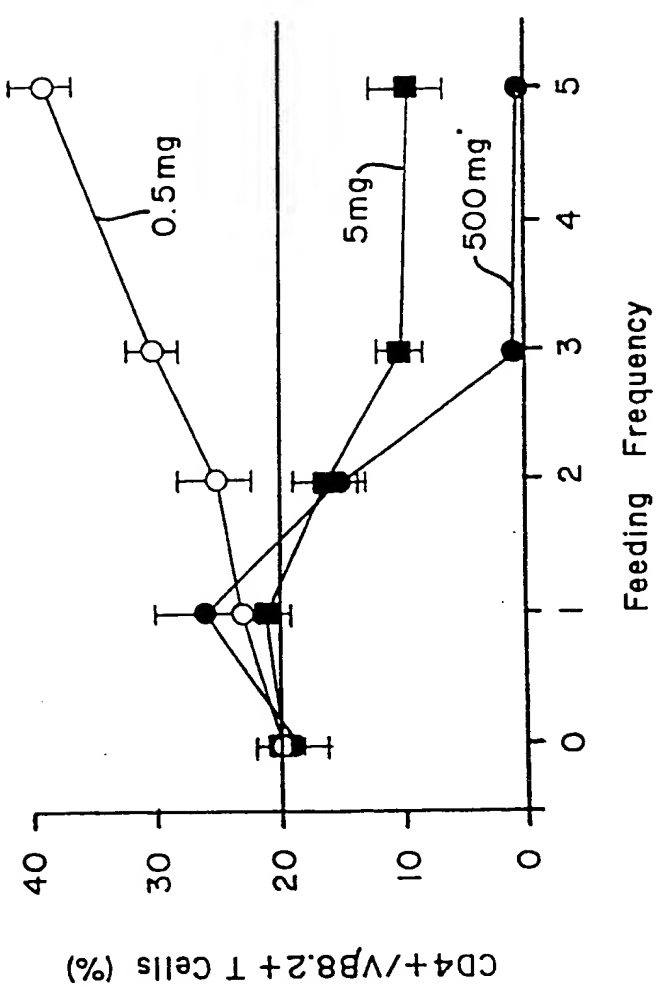

1.0
0.8
0.6
0.4
0.2
Anti-OVA Antibodies Bound (405 nm)
0
5
10
15
20
25
Days of Continuous Exposure to OVA

FIG. 5A


0.5mg
5mg
500mg
Feeding Frequency
0
1
2
3
4
5
40
30
20
10
0
CD4+/Vβ8.2+ T cells (%)

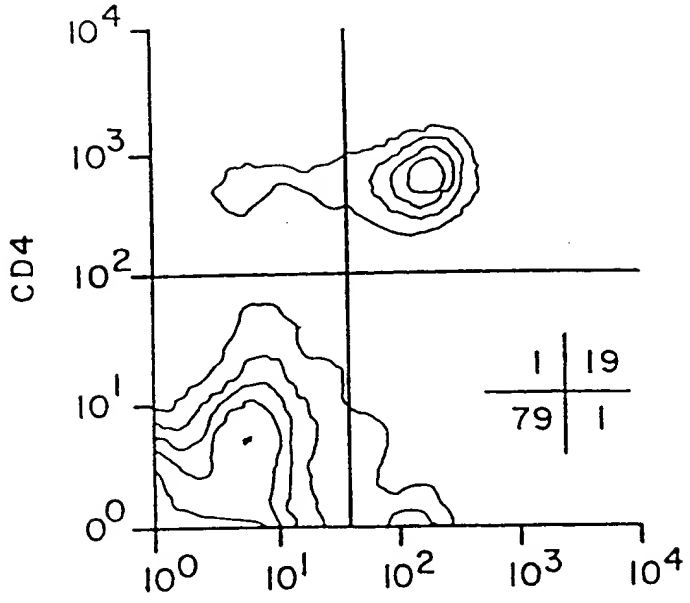

CD4
10^4
10^3
10^2
10^1
10^0
1 | 19
79 | 1
10^0 10^1 10^2 10^3 10^4
T Cell Receptor VβB 8.2

FIG. 5B

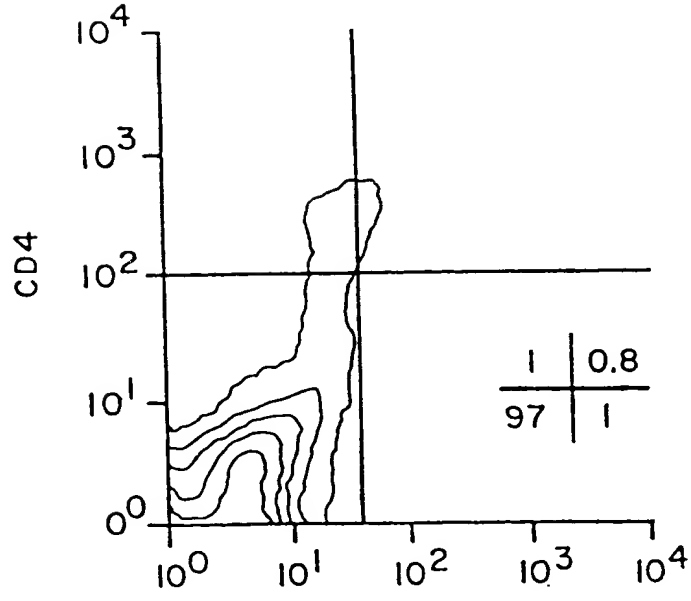
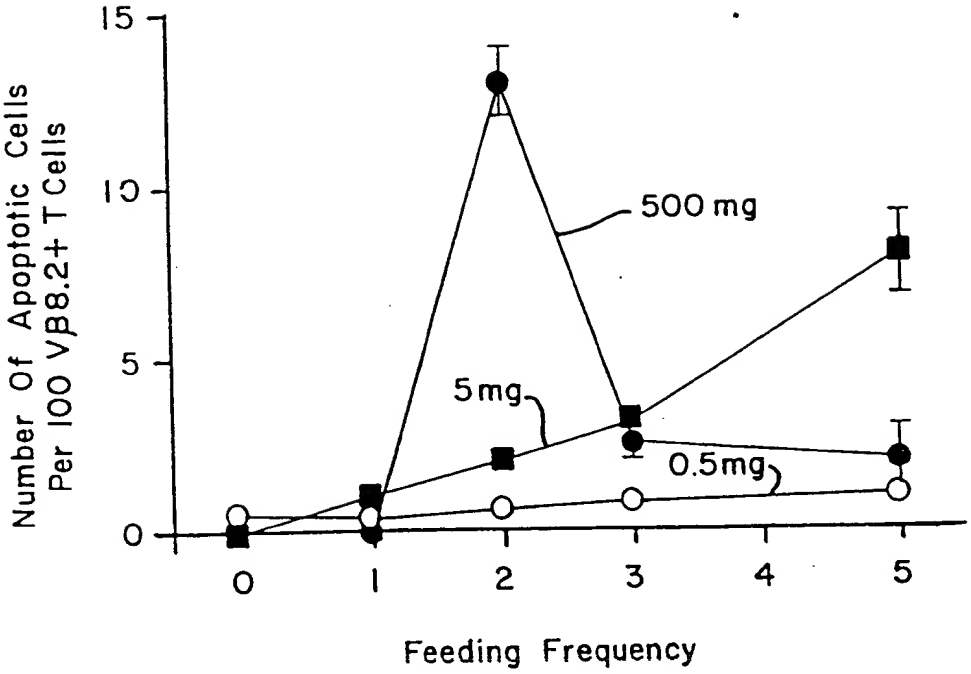

CD4
10^4
10^3
10^2
10^1
10^0
1 | 0.8
97 | 1
10^0 10^1 10^2 10^3 10^4
T Cell Receptor VβB 8.2

FIG. 5C

FIG. 6A


15
10
5
0
Number Of Apoptotic Cells
Per 100 Vβ8.2+ T Cells
500 mg
5mg
0.5mg
0
1
2
3
4
5
Feeding Frequency

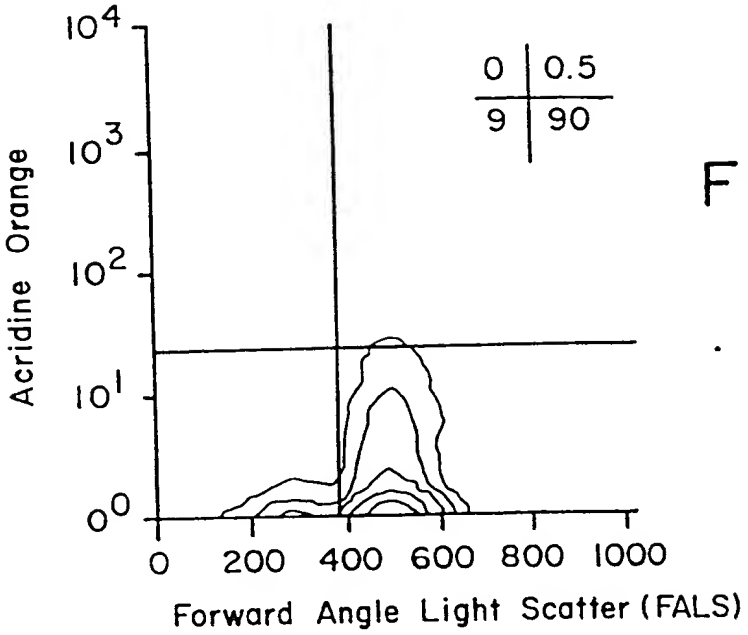

0 | 0.5
9 | 90
Acridine Orange
10^4
10^3
10^2
10^1
10^0
0 200 400 600 800 1000
Forward Angle Light Scatter (FALS)

FIG. 6B

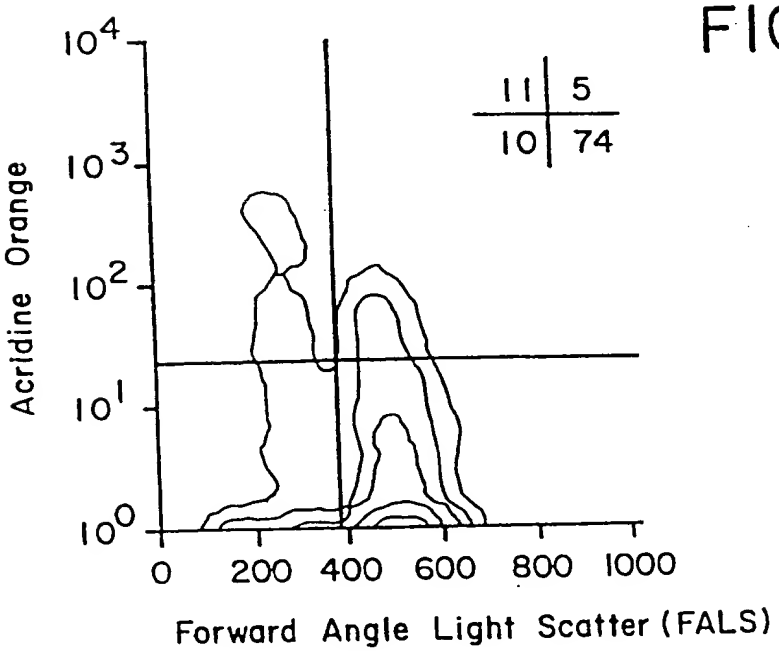

11 | 5
10 | 74
Acridine Orange
10^4
10^3
10^2
10^1
10^0
0 200 400 600 800 1000
Forward Angle Light Scatter (FALS)

FIG. 6C

FIG. 7A

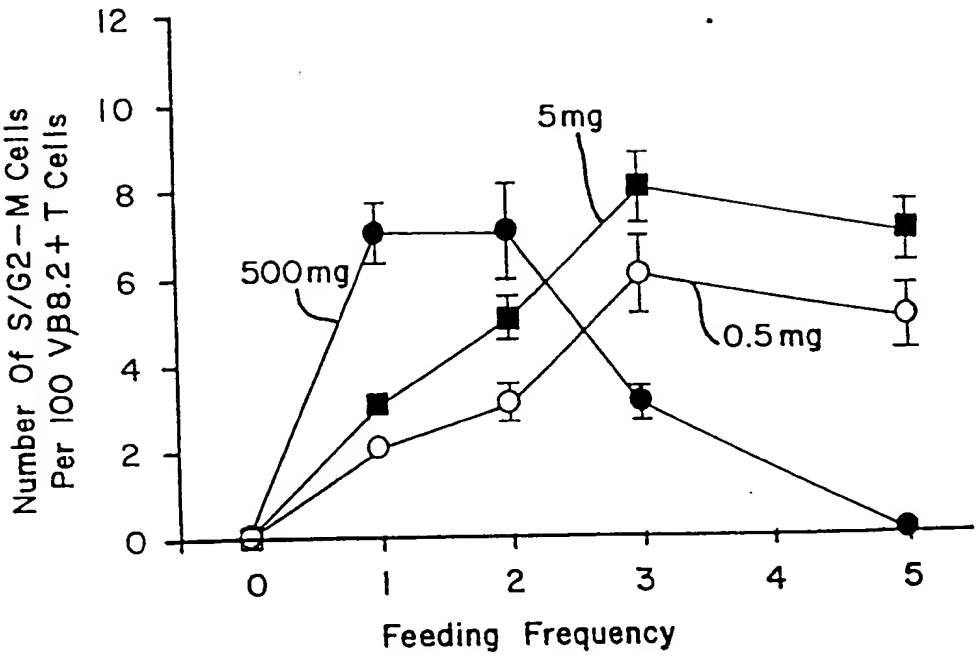

Number Of S/G2-M Cells
Per 100 VB8.2+ T Cells
12
10
8
6
4
2
0
5mg
500mg
0.5mg
0
1
2
3
4
5
Feeding Frequency

FIG. 7B

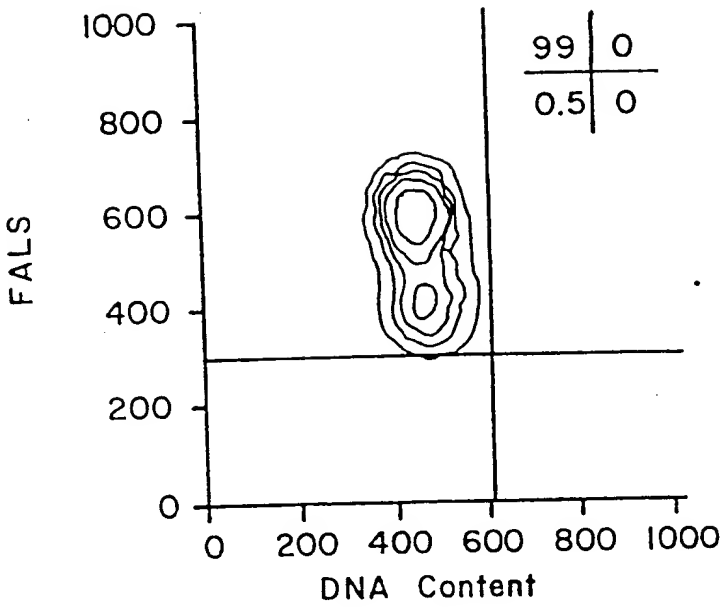

99 | 0
0.5 | 0
1000
800
600
400
200
0
FALS
0 200 400 600 800 1000
DNA Content

FIG. 7C

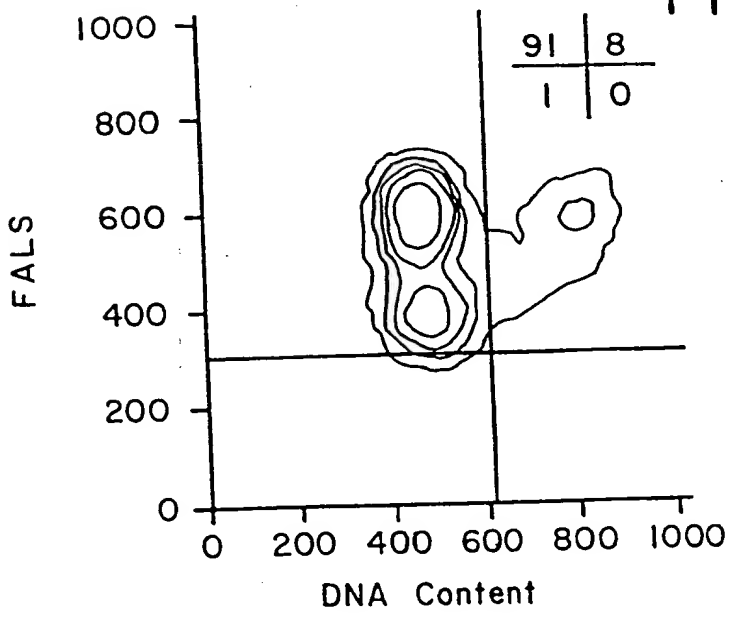

91 | 8
1 | 0
1000
800
600
400
200
0
FALS
0 200 400 600 800 1000
DNA Content

FIG. 8A

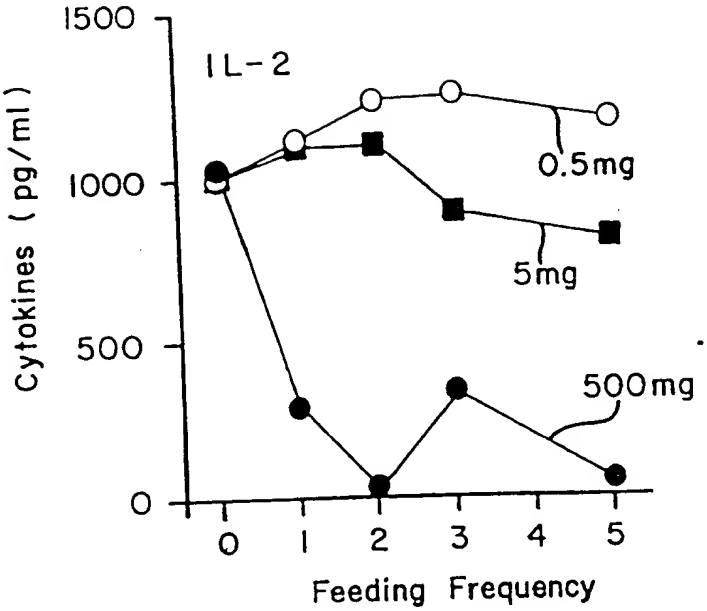

1500
1000
500
0
IL-2
Cytokines (pg/ml)
0.5mg
5mg
500mg
0 1 2 3 4 5
Feeding Frequency

FIG. 8B

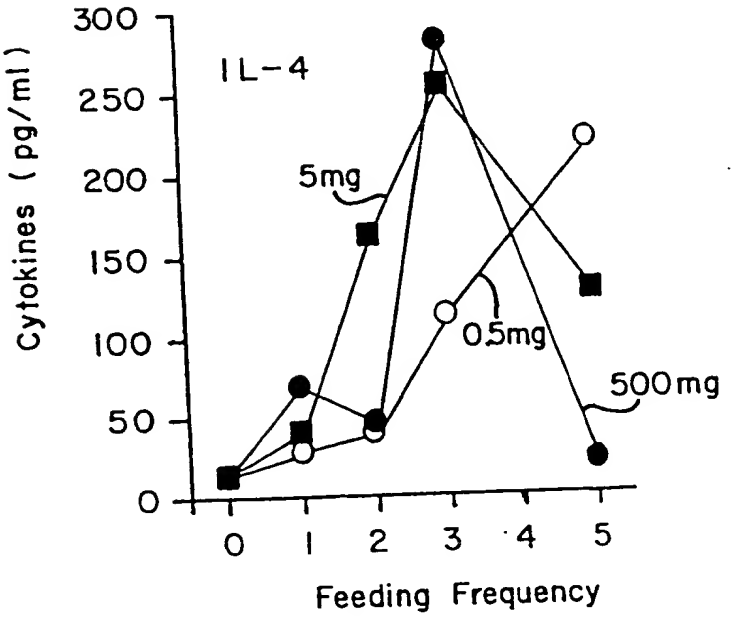

300
250
200
150
100
50
0
IL-4
Cytokines (pg/ml)
5mg
0.5mg
500mg
0 1 2 3 4 5
Feeding Frequency

FIG. 8C

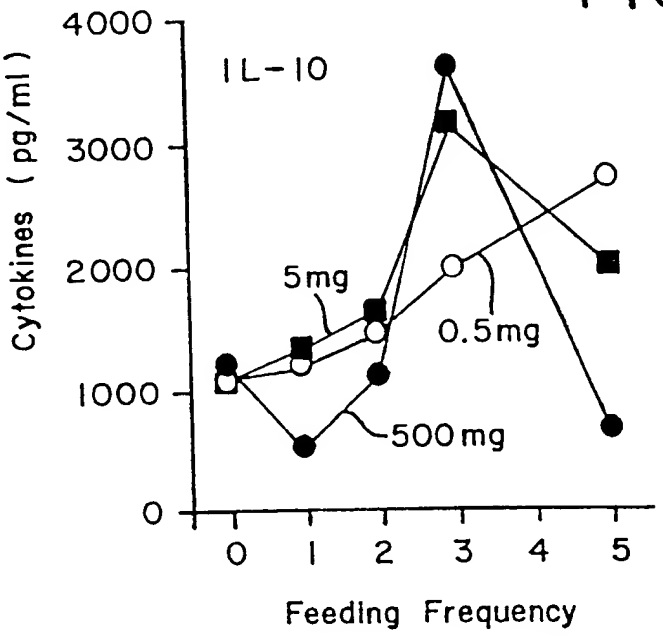

IL-10
Cytokines (pg/ml)
4000
3000
2000
1000
0
5mg
0.5mg
500mg
0 1 2 3 4 5
Feeding Frequency

FIG. 8D

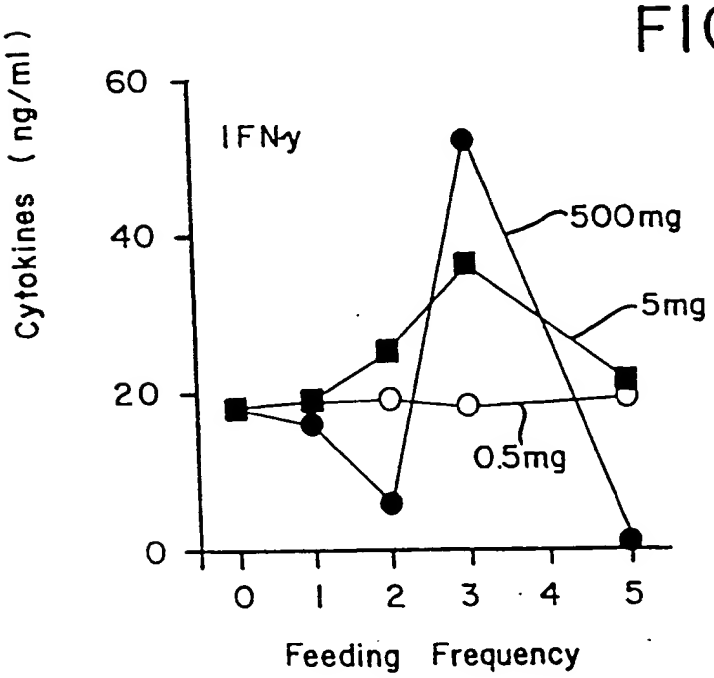
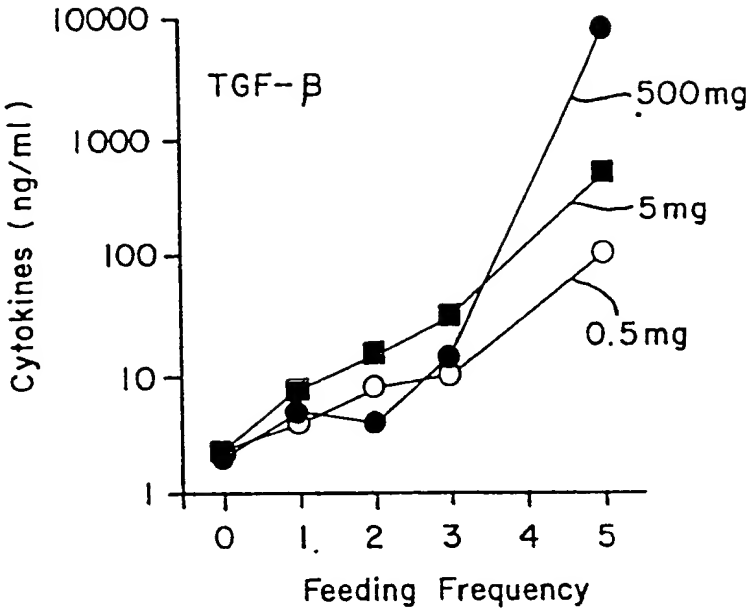

IFN-γ
Cytokines (ng/ml)
60
40
20
0
500mg
5mg
0.5mg
0 1 2 3 4 5
Feeding Frequency

FIG. 8E


10000
1000
100
10
1
TGF-β
500mg
5mg
0.5mg
Cytokines (ng/ml)
0
1
2
3
4
5
Feeding Frequency

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10386

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/00, 39/38, 38/28; C07K 1/00, 14/00, 17/00

US CL : 424/184.1, 185.1; 514/3; 530/350, 868

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 185.1; 514/3; 530/350, 868

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPIDS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ZHANG et al. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. Proc. Natl. Acad. Sci. USA. November 1991, Vol. 88, pages 10252-10256, see entire document.	1-3, 10, 11 ---------- 4-9, 12
X	GARSIDE et al. T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. Journal Immunology. 01 June 1995, Vol. 154, pages 5649-5655, see entire document.	1-3, 10, 11
Y	WEINER et al. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. Science. 26 February 1993, Vol. 259, pages 1321-1324, see entire document.	4-9, 12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 AUGUST 1996	17 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer EVELYN RABIN Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10386

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 91/01333 A1 (AUTOIMMUNE, INC.) 07 February 1991 (07/02/91), see entire document.	4-9, 12
X	WO 93/15750 A1 (HAYNES) 19 August 1993 (19/08/93), see entire document.	13

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(54) Title: MONOCLONAL ANTIBODY SPECIFIC FOR βA4 PEPTIDE

(57) Abstract

The instant invention provides for monoclonal antibody which is specific for the βA4 peptide, and in particular the free C-terminus of βA4 "1-42" but not "1-43", and stains diffuse and fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. The instant invention further provides for antibody fragments and constructs thereof which have the same binding specificity. The instant invention also provides for methods of diagnosis, screening and therapeutics for treating unique forms of βA4 peptide, using the antibodies of the instant invention.

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M noclonal Antibody Specific for βA4 Peptide

Field of the Invention

This application relates to Alzheimer's disease, particularly, to a monoclonal antibody specific for the βA4 peptide derived from Amyloid Precursor Protein, cells which produce such antibody, methods of generating such monoclonal antibodies, and to methods for using such antibodies in diagnostics and therapy.

Background

Alzheimer's Disease (AD) is an irreversible progressive neurodegenerative brain disorder. Over the course of several years the progression of AD leads to memory loss, dementia, and finally death. Currently, it is the fourth leading cause of death in the United States, accounting for approximately 100,000 deaths annually. Typically, AD affects primarily the elderly and is therefore, with the aging of modern society, expected to be an increasing health concern in the near future. Soon after the onset of the disease, patients require assistance around the clock. This represents a tremendous psychological as well as financial problem for our society. At present, no proven means for diagnosis, prevention, treatment, or cure of AD exist.

Neuropathologically, AD is characterized by massive neuronal cell loss in certain brain areas, and by the deposition of proteinaceous material in the brains of AD patients. These deposits are the intracellular neurofibrillary tangles and the extracellular ß-amyloid plaques. The major protein component of the ß-amyloid plaque is the βA4 peptide. Sequence analysis of purified ß-amyloid plaque material and mass spectrometry showed that the maximum length of the βA4 peptide is 43 amino acids. Typically, however, species of the peptide can also end either at position 40 or position 42 (Miller *et al.*, 1993, *Arch. Biochem. Biophys.* 301:41-52). Similarly, at the N-terminus, a certain raggedness can be observed, leading to several different forms of

the peptide, starting mainly at position 1, 4 or 11 (Miller *et al.*, 1993).

Molecular cloning revealed that the βA4 peptide is derived from a much larger precursor protein termed the "Amyloid Precursor Protein" (APP) (Kang *et al.*, 1987, *Nature* 325:733-736) (Figure 1). Figure 1 illustrates the Amyloid Precursor Protein (APP) which is a transmembrane (Tm = membrane region) protein where the N-terminus is located extracellular and the C-terminus is located intracellular (cytoplasmic). βA4 is partially embedded into the membrane. Several alternatively spliced isoforms have been described, which undergo extensive post-translational modifications (Selkoe, 1994, *Ann. Rev. Neurosci.* 17:489-517). The βA4 sequence itself is located partially on the extracellular side and extends partially into the transmembrane region (Figure 2). Figure 2 (SEQ ID NO:3) illustrates the βA4 sequence, shown (encircled area) extending with its C-terminal end into the transmembrane region (Tm, boxed area) and the N-terminal end located in the extracellular part. Asterisks indicate the location of familial mutations in the APP gene; they are either flanking the βA4 sequence, or are centered in the middle portion of the βA4 sequence. The three major cleavage sites (α, β and γ) in APP are indicated. Release of βA4 was therefore postulated to occur through the proteolytic action of one or more proteases on the N-terminus (ß-cut) and on the C-terminus (γ-cut) of the peptide (Figure 2) (Selkoe, 1994). The main event during the secretion of APP is at the α-cut (position 16/17 of βA4 "1-42"). This secreted APP molecule (αAPPs) contains the first 16 amino acids of the βA4 sequence at its carboxyl end. The remaining cell-associated APP fragments (so called C-terminal fragments (CTF's)) contain the C-terminal portion of the βA4 sequence and extend to the cytoplasmic region of APP. Therefore, this proteolytic cut results in fragments which may not be processed in such a way that directly or indirectly leads to amyloidogenic fragments (non-amyloidogenic processing) (Selkoe, 1994).

Recently, it was demonstrated that cell lines which express large amounts of

APP through a stably transfected APP cDNA construct produce high picomolar to low nanomolar amounts of βA4 and release it rapidly into the medium (Shoji *et al.*, 1992, *Science* 258:126-129). It has also been found that virtually every primary cell culture and cell line releases βA4 constitutively (Busciglio *et al.*, 1993, *PNAS USA* 90:2092-2096). Additionally, healthy controls as well as Alzheimer patients have been shown to have low nanomolar amounts of βA4 in sera and CSF (Seubert *et al.*, 1992, *Nature* 359:325-327). The majority of the detected soluble βA4 species in these body fluids and conditioned media was βA4 "1-40", which is not truly reflective of the overall composition found in β-amyloid plaque depositions. The notion that the production and subsequent release of βA4 is sufficient and therefore responsible for the buildup of β-amyloid plaques in the brains of AD patients could therefore no longer be maintained; other factors must contribute to the deposition of β-amyloid plaques. One straightforward hypothesis is that acute or chronic overproduction of βA4 causes the increased amyloid load observed in AD

The finding that specific point mutations in and around the βA4 region of the APP gene are linked with certain familial Alzheimer's disease (FAD) cases showed unequivocally that the APP gene is a "disease gene" (Goate *et al.*, 1991, *Nature* 349:704-706; Murrell *et al.*, 1991, *Science* 254:97-99; Levy *et al.*, 1990, *Science* 248:1124-1126; Carter *et al.*, 1992, *Nature Genetics* 2:255-256). In families in which AD is inherited dominantly with a specific age of onset, the point mutations in the APP gene are necessary and sufficient to cause AD. Although the vast majority of Alzheimer disease cases are sporadic and probably multifactorial, these familial APP mutations can teach us a great deal about amyloidogenesis, i.e., the generation of the small βA4 peptide from the larger precursor and its subsequent deposition in β-amyloid plaques.

The double mutation at APP codon 670/671 (the "Swedish variant", at the N-terminus of βA4 in APP) causes a 5 to 8 fold higher release of βA4 in cell cultures

stably transfected with that mutated APP cDNA (Figure 2) (Citron *et al.*, 1992; Cai *et al.*, 1993). It is conceivable that this double point mutation leads to an increased turnover of APP due to increased proteolysis at the ß-cut, which in turn leads to a higher level of released ßA4. Increased amounts of ßA4 monomers, as demonstrated by transfection studies with the "Swedish mutation", can explain the faster kinetics of ßA4 aggregation to ß-amyloid plaques in these families.

Another FAD mutation lies C-terminal of ßA4 at position 717 ("London variant") and does not affect the level of released ßA4 in tissue culture (Figure 2). It was recently demonstrated that this 717 mutation changes the "1-40/1-42" ßA4 ratio (Suzuki *et al.*, 1994, *Science* 264:1336-1340). Although it is not clear at the moment how the generation of the C-terminus of ßA4 occurs, since this part is embedded in the transmembrane region, it is tempting to hypothesize that the "London mutation" affects the proteolytic breakdown of APP to ßA4. Possibly, this point mutation interferes with the cleavage fidelity of the responsible protease at the γ-site. ßA4 1-40 exhibits among other things a dramatic difference in its solubility in aqueous solutions when compared to ßA4 1-42 (Burdick *et al.*, 1992, *JBC* 267:546-554). The latter is virtually insoluble in water, whereas 1-40 is water soluble up to several mg/ml in vitro. Minor amounts of the longer 1-42 form can enhance precipitation of 1-40 in vitro dramatically. A slightly higher proportion of the longer 1-42 ßA4 species would explain the early onset deposition of ßA4 to ß-amyloid plaques in patients with this "London mutation". The proportion of the 1-42 species to the shorter more soluble 1-40 species may also be one of the critical factors in the sporadic AD cases (i.e. cases where no genetic predisposition was identified). Monoclonal antibodies which specifically bind to the 1-42 species, are therefore useful to investigate the production and presence of ßA4 species ending at amino acid position 42, and can be used as a diagnostic indicator of abnormal species present in AD.

Recent biochemical analyses with one antibody which recognizes ßA4 ending at

position 40, and one antibody recognizing βA4 species extending to position 42 or further, showed that the contribution of the longer βA4 species might be critical for the onset of the disease (Suzuki *et al.*, 1994). However, the monoclonal antibody of Suzuki does not distinguish between βA4 1-42, 1-43, and longer βA4 species. This is also the case for another reported monoclonal antibody 2G9 (Yang *et al.*, 1994, *Neuro Report* 5:2117-2120). Therefore, in order to avoid this crossreactivity, antibodies which are specific for βA4 species ending at position 42 to the exclusion of the other forms would be very useful in order to avoid crossreactivity with membrane associated C-terminal APP fragments, which are typical cellular products not necessarily associated with the β amyloid plaques.

One monoclonal antibody recognizing βA4 1-42 has been described (Murphy *et al.*, 1994, *Am. J. Path.* 144:1082-1088). However, the βA4 1-43 peptide species was not used in these studies, thus it is not known what the exact specificity of this monoclonal antibody would be in response to the 1-43 peptide. Competition studies were only performed with βA4 peptides ending at position 40 ("1-40"), and position 44 ("1-44") and beyond with this antibody. Importantly, the antibody was reported to stain diffuse amyloid, fibrillar amyloid, intraneuronal and extraneuronal neurofibrillary tangles, but *not* vascular amyloid.

An in vitro biochemical diagnostic test for Alzheimer's disease in its early stages as well as a means of screening for at-risk AD individuals is not available. The current diagnosis of AD requires a detailed clinical evaluation which cannot give clear answers until significant symptoms of dementia and memory loss are manifested. In view of the research referred to above, βA4 1-42 represents a preclinical marker for AD. Thus, identifying the level of or the buildup of βA4 1-42, or other residue 42 terminating species, and how this may progress during the course of the disease, and how it is distributed in the brain, will provide valuable insights into monitoring the course of, as well as for specific diagnosis and possible treatment of AD.

It would be useful for preparing diagnostic tests, therapeutics and for monitoring assays of AD, to have a monoclonal antibody which, in contrast to the specificity of presently available antibodies (crossreactive with 1-43; reported not to stain vascular amyloid), does stain vascular amyloid and is specific for βA4 peptide ending at residue 42 , and therefore extends the diagnostic capabilities of the art, i.e. one that recognizes the free C-terminus of βA4 1-42 and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. Such an antibody is the subject of the present application.

Summary of the Invention

The instant invention provides for monoclonal antibody which is specific for the βA4 peptide, and in particular, the C-terminus of βA4 "1-42" and stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid. In particular, the instant invention provides for monoclonal antibody which is specific for all βA4 peptides in which the C-terminus is residue 42 of the βA4 amino acid sequence. The instant invention further encompasses antibody fragments and constructs thereof which have the same binding specificity. The instant invention encompasses in particular the monoclonal antibody known as "Mab 369.2B" and is produced by the cell line "369.2B" which has been deposited under the Budapest Treaty with the American Type Culture Collection (ATCC) on January 26, 1995, and has been assigned the ascension number HB11829. The instant invention encompasses the use of the monoclonal antibody of the instant invention in diagnostic, purification, and therapeutic uses.

Thus one embodiment of the instant invention encompasses a monoclonal antibody specific for βA4 peptide ending at position 42, wherein said antibody binds to diffuse amyloid, fibrillar amyloid, vascular amyloid, and neurofibrillary tangles. In one particular embodiment, the instant invention provides for a monoclonal antibody which is specific for the C-terminal amino acids of the βA4 1-42 peptide. In the most

preferred embodiment the instant invention encompasses a monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829. A preferred embodiment of the instant invention also encompasses a cell which is identified by the ATCC ascension number HB 11829. In a further embodiment of the instant invention, an immunologically reactive fragment of the monoclonal antibody of the instant invention which is capable of the same binding as the monoclonal antibody of the instant invention, is encompassed.

The instant invention also provides for methods of generating βA4 specific antibodies which recognize the free C-terminal residue 42. The instant invention also provides for methods for detecting the presence of βA4 peptides ending at position 42, in tissue comprising contacting a tissue sample with monoclonal antibody of the instant invention, by detecting the presence of monoclonal antibody in a selective fashion. The instant invention also provides for methods for selective purification of βA4 peptides ending at position 42, comprising contacting a sample to be purified with monoclonal antibody of the instant invention, separating the βA4 peptide from the sample to be purified, and isolating the βA4 peptide. In a further embodiment, the instant invention provides for methods for detection of βA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with monoclonal antibody of the instant invention, and detecting the presence of βA4 peptides.

Thus the instant invention also provides for methods for the prevention of aggregation of βA4 peptide by administering monoclonal antibody of the instant invention. In a preferred embodiment the monoclonal antibody is identicle to 369.2B, or is an immunologically active fragment with equivalent binding specificity thereof. The instant invention thus provides a means for detecting the presence of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. As well as a means for preventing the aggregation of βA4 peptide

comprising an immunologically reactive fragment of the monoclonal antibody of the instant invention. The instant invention provides a means for detecting and monitoring the level of βA4 peptide in tissue or fluid samples (e.g. blood, other body fluids, tissue sections, biopsy tissues etc.). In a preferred embodiment, the βA4 peptide being detected, monitored, inhibited or purified is a βA4 peptide with a free carboxy terminal amino acid residue being residue number 42 of the βA4 peptide amino acid sequence.

All references to publications and patent documents in the prior or subsequent sections are hereby incorporated by reference in the entirety. Specific preferred embodiments of the present invention will become more evident from the following more detailed description of certain preferred embodiments and the claims.

Brief Description of the Drawings

The invention will be more completely understood from a consideration of the following detailed description, taken in conjunction with the drawings, in which:

Fig. 1 is a schematic showing the βA4 portion of the Amyloid Precursor Protein (APP), its location relative to the cell membrane, and the α, β and γ cleavage sites;

Fig. 2 shows the βA4 portion of APP, its position relative to the transmembrane region of a cell, and the three major cleavage sites (α, β and γ) in APP;

Fig. 3 is a diagram of the clone pGK003 which was used to generate the βA4 1-42 peptide;

Fig. 4A shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled βA4 in a wheat germ system;

Fig. 4B shows SDS-PAGE on a 16 % Tris/Tricine gel, of in vitro translated radioactively labelled βA4 from wheat germ system, immunoprecipitated with MAb 286.8A;

Fig. 5 is a graph showing immunoprecipitation of in vitro translated βA4 (IVT βA4) with 286.8A;

Fig. 6 is a diagram of the peptides used to generate the immune response (immunogen) and to screen the sera of mice;

Fig. 7 is a graph showing immunoprecipitation of in vitro translated βA4 vs. antibody concentration; -○- 286.8A, -△- 369.2B, -□- 369.6;

Fig. 8 is a graph showing the % of various βA4 sequences immunoprecipitated by MAb 369.2;

Fig. 9 is a graph showing epitope mapping of MAb 369.2 by competitive assay, with -■- being 35-42(OVA) (Ovalbumin coupled 35-42 βA4 peptide), -□- being 1-42 βA4 peptide, and -♦- being 1-40 βA4 peptide;

Fig. 10 is a photograph showing the binding of MAb 369.2B to vascular amyloid and other plaques with various morphologies.

Detailed Description of the Invention

The ß-amyloid depositions in Alzheimer's disease brains are made up mainly of βA4 peptides which show both N- as well as C-terminal heterogeneity. The major C-terminal species, identified by peptide sequencing of ß-amyloid purified from postmortem brain material, end either at position 40 or 42 of the βA4 peptide which is at most 43 residues long. In vitro experiments with synthetic βA4 peptides ending either at position 40 or 42 indicate profound physico-chemical differences. Previously, the distribution of these two βA4 species in postmortem tissue as well as their generation in vitro could not be addressed due to the lack of specific antibodies against the carboxy end capable of distinguishing between subspecies of βA4 peptide.

Recent evidence suggests that release of βA4 is a normal event in virtually every cell culture. Typically high picomolar to low nanomolar concentrations of βA4 can be measured in serum and cerebral spinal fluid (Seubert *et al.*, 1993). This finding was surprising because it had been assumed that the production of βA4 is a pathological event since βA4 is massively deposited as ß-amyloid plaques in the

cortical and hippocampal brain regions of Alzheimer disease patients. Detailed sequence analysis of the released βA4 from cell culture revealed that the major species end at position 40 (Selkoe, 1994). Amyloid plaques purified from postmortem brain show a slightly different picture: amyloid deposits of the congophilic amyloid angiopathy (CAA) are βA4 aggregates surrounding blood vessels and are predominantly βA4 1-40, whereas in contrast amyloid plaques cores (APC) which are present in the brain parenchyma and are not associated with blood vessels exhibit an N-terminal raggedness (starting most commonly at residues 1, 4 and 11) and end mainly either at position 40 or 42 (Glenner and Wong, 1984, *Biochem. Biophy. Res. Comm.* 120:885-890; Masters *et al.*, 1985, *PNAS USA* 82:4245-4249; Miller *et al.*, 1993). Occasionally, longer species ending at 43 or extending even further have been described (Miller *et al.*, 1993). Because the length of the hydrophobic C-terminus is critical for the ability of the peptide to self-aggregate in vitro (Burdick *et al.*, 1992; Jarrett *et al.*, 1993, *Biochem.* 32:4693-4697), it is entirely possible that the two distinct pathological aggregates, APC and CAA and other vascular ß-amyloid plaques, can be explained by the differing properties of the two species 1-40 and 1-42. This could also be the case for the so called "diffuse plaques" (Selkoe, 1994) which are seen frequently in brains of aged humans and are not associated with AD, however, have been proposed to be precursors of fibrillar ß-amyloid deposits. A non-fibrillar aggregation of βA4 has been suggested for these structures. It is therefore of primary importance to determine the tissue specific production of these longer βA4 species (i.e., those ending at position 42) and their pathological appearance in brains of AD patients. Recently three reports have been published where antibodies have been described, which distinguish 1-42 and 1-40 species of βA4 (Suzuki *et al.*, 1994; Murphy *et al.*, 1994; Yang *et al.*, 1994). Unlike the antibodies of the instant invention, the antibodies reported by Suzuki *et al.*, and Yang *et al.*, crossreact in a significant degree with both the 1-43 and 1-42 species of βA4 peptide. The antibody of Murphy *et al.*, while not

tested for binding with the 1-43 species of βA4 peptide, exhibits a different tissue binding pattern than the antibodies of the instant invention, and thus must recognize a different, or modified epitope from that recognized by the antibodies of the instant invention.

Positions 29 through 42 of the βA4 peptide lie entirely within the putative transmembrane region of the Amyloid Precursor Protein and are hydrophobic in nature (Miller *et al.*, 1993). Synthetic peptides to the C-terminal sequences in this region must overcome the ability of the 34-42 sequence to form an unusually stable ß-structure which is virtually insoluble in water and strong denaturants (Halverson *et al.*, 1990, *Biochem.* 29:2639-2644) if they are to be used to elicit good immune responses against soluble βA4. We designed a hydrophilic spacer five residues long which would overcome those insolubility problems and also extend the presumed epitope away from the proximity of the carrier. To reduce the likelihood of cross-reactivity with the shorter but major βA4 species, 1-40, we chose a minimal peptidyl epitope of 8 residues corresponding to positions 35-42 of the βA4 sequence. The entire synthetic sequence designed in this way was coupled by a free sulfhydryl group on a N-terminal cysteine residue to KLH (keyhole limpet hemocyanin).

Successful use of spacers and hydrophilic residues in the production of antipeptide antibodies is well documented as is the use of hydrophilic structures to bring insoluble haptens into solution for conjugation (McMillan *et al.*, 1983, *Cell* 35:859-863; Makela and Seppala, 1986, in Handbook of Experimental Immunology, Volume 1: Immunochemistry, Wier, D.M., editor. Blackwell Scientific Publications, Oxford. pp 3.1-3.13). The success of this method in producing specific antibodies may at least in part be attributed to the presence of a free charged carboxyl terminal, especially in context of a hydrophobic sequence, as terminal residues on peptide antigens give rise to significant proportions of antipeptide antibodies (Gras-Masse et al., 1985, in Synthetic Peptides in Biology and Medicine, Alitalo, K. *et al.*, editors,

Elsevier, Amsterdam, p 105). This, along with the selective and novel use of a minimal βA4 sequence used as an immunogen maximized the probability of producing an antibody which could distinguish between βA4 species ending at positions 42 with those that do not. Although peptide competition studies did not fine map the antigenic determinant, βA4 sequences other than 1-42 were not effective in inhibiting binding. The fact that 1-42 did not totally compete with ^{35}S-methionine-labelled in vitro-translated βA4 may be due either to the particular properties of the molecule itself or to the fact that the 35-42 peptide immunogen was presented in the context of a specific spacer and/or carrier, or that a 200-1000 fold excess of unlabelled peptide is not enough to quench the signal. Non-specific effects of N-terminal residues on antigenic activity are also well documented (Benjamini *et al.*, 1968, *Biochem.* 7:1261-1264).

The intriguing finding that 25-35 actually enhances the ability of 369.2B and other antibodies to bind to βA4 may be due to a peculiar interaction between the abstracted peptide and the full length βA4 sequence itself. Residues 26-33 are believed to exist as a random coil in aqueous solution (Halverson *et al.*, 1990) and may be able to interact with soluble βA4 in such a way that makes the C- terminus more accessible to the binding sites on antibodies.

The highly specific antibody of the instant invention, of which 369.2B is a particular example was raised against a synthetic βA4 peptide having residues 35-42, and does not recognize the shorter βA4 species 1-40 in solution or on a solid phase. Furthermore, both 1-40 and 1-43 were unable to absorb out the antibody when used immunohistochemically. A secondary screening method with medium capacity through-put for the screening of hybridoma supernatants using radioactively labelled in vitro translated βA4 was applied so that antibodies culled from the primary screening could be further selected for their ability to immunoprecipitate soluble βA4. This method can be easily adapted to other proteins/antibodies of interest. The resulting

MAb 369.2B represents a superior tool to investigate the rol of βA4 peptides ending at position 42 in situ, postmortem tissue, transgenic animals, and the in vitro generation of βA4 peptides in established cellular βA4 production models, for diagnostic use, and for therapeutics.

The monoclonal antibody of the present invention represents an important tool needed to establish a diagnostic test kit. It permits one to measure/quantify the amount of the βA4 1-42 or derivatives thereof (e.g. 4-42 species, and other truncated forms with the "42" carboxy end) in human body fluids (CSF, serum, urine etc.) or tissues. It can also be used to study the distribution pattern of 1-42 or βA4 species ending in residue 42, in AD brains compared to healthy controls. Its exceptional high avidity makes it a superior and novel tool for such testing. The monoclonal antibody here disclosed can also be used in biological model systems such as transfected cell cultures or animal models (transgenic mice), designed to measure and/or influence the presence and/or production of βA4 species ending in amino acid 42. These model systems represent means to identify selective modulators of the production of βA4 ending in amino acid position 42 of the βA4 in biological systems. The antibodies of the instant invention provide for methods of preventing aggregation of βA4 peptide because the specificity of the antibody will allow for the specific interference with the free C-terminal residue, thereby interfering with and disrupting aggregation that may be pathogenic in AD.

Surprisingly, the antibody of the present invention differs from that of the prior art in that it stains diffuse and fibrillar amyloid, neurofibrillary tangles, and vascular amyloid while being specific for the βA4 peptide free C-terminal residue 42. This unique binding pattern shows that the antibody of the instant invention recognizes a different epitope from that of the prior art, and that the tissue distribution or accessibility of the βA4 peptide recognized by the antibody of the instant invention is also different. Further, the instant invention provides for monoclonal antibody which

can precipitate the βA4 peptide out of solution, which was not demonstrated by the antibodies of the prior art. Thus the instant invention provides for unique monoclonal antibodies which recognize a unique subset of βA4 species which has a distinct tissue distribution that is most likely a better diagnostic indicator than what was previously available, and a unique target for therapeutic intervention.

Thus the instant invention provides for antibodies, antibody fragments and constructs thereof which are specific for the βA4 species of peptide where the C-terminal ends at residue 42. The instant invention also provides for the use of such antibodies, binding fragments and constructs thereof in diagnostic, analytic, therapeutic, and biochemical purification methods which employ the binding specificity of the instant monoclonal antibodies and their use within pharmaceutical formulations.

The following examples will further explain the instant invention and are shown by way of illustration, and not by way of limitation. The following examples illustrate certain aspects of the above-identified methods and compositions as well as advantageous results.

Example 1: βA4 Peptide Expression System

Preparation of plasmid pGK002

General cloning and molecular biology procedures are found for example in Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning 2nd edition, Cold Spring Harbor Lab Press. Plasmid pMTI-26, which is a Bluescript KS clone containing 2.415 kb (kilobase pairs) of the APP sequence with a TAG stop codon followed by a BamHI site placed in frame by site-directed mutagenesis after the 42nd amino acid codon of the βA4 region, was modified by excising a 1.8 kb Xba I/Bgl II fragment and religating the plasmid after filling in the ends. The resulting construct, designated pGK002, places the consensus containing initiation codon of the βA4 sequence immediately downstream of the Bluescript T7 promotor.

Preparation of plasmid pGK003

Plasmid pGK003 (Fig. 3), used in all of the wheat germ in vitro translations of βA4 to be described below, was made by subcloning a 590 bp (base pair) NotI/Xho I fragment from pGK002 containing the entire human βA4 sequence with the mutagenized stop/Bam HI into a pSP64 polyA vector (Promega Corp.). In preparing this plasmid, pGK002 was digested with Not I and Xho I and the resulting 590 bp fragment was filled in with Klenow, isolated, and ligated with pSP64polyA linearized with Sma I. Figure 3 is a diagram of Clone pGK003. The open reading frame of βA4 1-42 is expressed in vitro from the bacterial SP6 promoter. The 3' untranslated (3'-UT) region of APP is shown in black.

Example 2: In Vitro Transcription and Translation of pGK003

Plasmid pGK003 was linearized with EcoRV and complete digestion was confirmed by agarose gel electrophoresis. The sample was extracted twice with phenol/chloroform, followed by two chloroform extractions and ethanol precipitation. The resulting pellet was washed once in 70% ethanol, partially dried under vacuum, and resuspended in TE at a concentration of 1 µg/µl.

In vitro transcripts using linearized templates at 30 µg/ml were prepared in 80 mM HEPES-KOH (pH 7.5) buffer containing 16 mM $MgCl_2$, 2 mM spermidine, 40 mM DTT, 3 mM ATP/CTP/GTP/UTP, 800 units/ml RNAsin Ribonuclease Inhibitor (Promega Corp.), 5 units/ml Yeast Inorganic pyrophosphatase (Sigma Corp.), and 1800 units/ml SP6 RNA polymerase (Promega Corp.). The reaction mixture was kept at 37°C for 4 hrs. The resultant transcript was verified by electrophoresis through a 1.2% agarose/TBE/EtBr gel with denatured samples (65°C x 10 min).

Transcripts were translated using Wheat germ extract (Sigma Corp.). Briefly, transcripts were heated (65°C x 10 min), mixed with the wheat germ extract containing KAc, RNAsin, and a methionine-minus amino acid mixture, and translated at 25°C for

1 hr in the presence of ^{35}S-labelled methionine (Amersham). Translation of a 4 kD (kilo dalton) βA4 protein was verified by SDS-PAGE using 16% Tris/Tricine gel (Novex). Gels were fixed and proteins visualized fluorographically using a commercial system, "Amplify" (Amersham).

The incorporation of label into in vitro translated βA4, which contains one methionine residue per molecule, was determined by gel slicing. 2 mm slices were solubilized in 1 ml of 30% hydrogen peroxide, 0.75M NH_4OH overnight at 37°C. Next a 10 ml volume of "Ready Value" scintillation cocktail (Beckman) was added and DPMs (Decay per minute) determined using a Beckman LS6000IC scintillation counter in the auto DPM mode. Typical reactions produce ~250 ng βA4/ml, or ~56 nM.

In vitro transcription followed by translation of the βA4 clone, pGK003, in a wheat germ system resulted in a single 4 kD protein product when visualized by fluorography on a 16% Tris-Tricine SDS polyacrylamide gel (Figure4A). Figure 4A shows the results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3: In vitro translated βA4 in a wheat germ system. The identity of this 4 kD product was confirmed by immunoprecipitation with βA4 specific antibodies (Figure 4B). Figure 4B shows results of SDS-PAGE on a 16 % Tris/Tricine gel. Lane 1: High MW markers. Lane 2: Low MW markers. Lane 3: In vitro translated βA4 from wheat germ system immunoprecipitated with MAb 286.8A. Transcription and translation of this as well as other βA4 clones in a combined reticulocyte lysate system (TnT) did not result in the same yield or purity of radioactively labelled βA4 (data not shown). This could be due to the short transcript or to the peculiar nature of the βA4 peptide itself.

The monoclonal antibody 286.8A, which was raised against crude peptide 1-42 and maps to region 3-8 of βA4, was able to precipitate this protein in a concentration-dependent manner (Figure 5). Figure 5 graphs this immunoprecipitation of in vitro translated βA4 (IVT βA4). Increasing amounts of IVT βA4 were

immunoprecipitated with a fixed amount of 286.8A (7.4 µg) in 100 µl in RIPA buffer.

Example 3: Immunogen and Screening Peptide Preparation

Peptides were prepared by standard Fmoc solid-phase procedures. (see for example Gras-Masse *et al.*, 1985).

Peptide #959, a 14 residue synthetic peptide having an N-terminal cysteine attached to a hydrophilic DGDGD spacer and residues 35-42 of human βA4 (resulting complete sequence: CDGDGDMVGGVVIA (SEQ ID NO: 1)), was coupled to a maleimide-activated KLH (Keyhole Limpet Hemocyanin) carrier using the commercially available "Imject" Activated Immunogen conjugation kit (Pierce). Briefly, 2 mg of peptide were dissolved in 200 µl of conjugation buffer and allowed to react at room temperature for 2 hrs with 2 mg of reconstituted maleimide-activated KLH. The conjugate was purified by gel filtration and used as an immunogen for monoclonal antibody production using standard protocols as described in Example 4.

Peptide #958, a 14 residue synthetic peptide having an N-terminal cysteine attached to a GGGGG spacer and residues 35-42 of the human βA4, (resulting complete sequence: CGGGGGMVGGVVIA (SEQ ID NO: 2)), was coupled to ovalbumin by dissolving 2 mg of peptide in 200 µl 6M guanidine, 0.01M phosphate pH 7.0 and conjugated as above to 2 mg of a reconstituted maleimide activated ovalbumin. The purified conjugate was used in ELISA-screening of monoclonal fusion products. Antibodies screened in this way are specific for the "35-42" determinant rather than the spacer, cysteine bridge or carrier portions of the immunogen.

Figure 6: Illustrates the peptide used to generate the immunresponse (the immunogen) and the peptide used to screen the sera of mice, as well as fusions, in the enzyme immunoassay plate (EIA) are shown. βA4 sequence 35-42 was synthesized together with a spacer and a C-terminal Cysteine, which was then used to couple it covalently via maleimide bridge to a large carrier molecule. Both, the spacer and the

carrier molecule in immunogen and screening peptide are different in order to select for βA4 sequence specific antibodies.

ELISA (Enzyme Linked Immunosorbant Assay)

Biotinylation of MAb

The N-hydroxysuccinimide ester of biotin is used to biotinylate monoclonal antibody 286.8A. The integrity of the reagent is first verified by watching it's spontaneous hydrolysis in the absence of primary amines: an 0.2 mg/ml solution of NHS-LC-Biotin (Vector Labs, Burlingame, CA) in PBS is monitored at 260nm over time. An OD260 of 1.0 after approximately 2 hrs (rising from and initial OD260 – 0.55) indicates the expected hydrolysis.

In the biotinylation reaction a 66:1 molar ratio of Biotin to monoclonal 286.8A at neutral pH has been found to give optimal results when the biotinylated 286.8A was tested in an Elisa format. NHS-LC-Biotin 0.6 mg in H_2O at a concentration of 0.1 mg/ml is added (within 5 min of dissolving) to 1 ml (2 mg) of 286.8A in PBS. Nucleophilic attack of the NHS ester is allowed to occur at 25°C for 4 hrs after which 10 mg of glycine in 50 µl H_2O is added to stop the reaction. The reaction is then placed over a 10 ml cross-linked dextran desalting column equilibrated to PBS and 0.5 ml aliquots are collected. The first peak representing the IgG peak is pooled and stored at 4°C until used.

Elisa procedures

Corning 25801 96-well Elisa plates are coated overnight at 4°C with 100 µl monoclonal 4G8 or other capture antibody at 5 µg/ml typically in H_2O or buffer. The plate is then washed with PBS containing 1% Triton X-100 in a Dynatech Ultrawash plus. Wells are then blocked for 90 min with 300 µl PBS containing 1% Triton X-100 and 1% Elisa grade BSA (Blocking Buffer). After washing antigen or

unknown diluted in blocking buffer is added to the wells in triplicates and incubated at room temperature for2 hrs. The plate is washed 2 times and 400 ng biotinylated 286.8A or other detecting antibody is added. After 30 min the plate is more extensively washed (2 times wash, 2 min soak, 2 times wash) and 100 µl preformed Avidin-Biotin-Alkaline Phosphatase Complexes (Vector Labs) are added. The plate is washed (2 times wash, 2 min soak, 2 times wash, 5 min soak, 4 washes) and MUP substrate added at 0.06 mg/ml 1x diethanolamine buffer. Plates are read in a Millipore Cytoflour after 15 min using a 360 nm excitation filter and a 460 nm emission filter.

Example 4: Generation of Monoclonal Antibodies

Balb/c mice were immunized with multiple I.P. inoculations of KLH conjugated peptide #959. Splenocytes from immunized animals were fused with the mouse myeloma AG8 using standard protocols (Wunderlich et al., 1992, J. Immunol. Methods 147:1-11). Supernatants from resultant hybridomas were screened for immunoreactivity to ovalbumin-coupled peptide #958 using standard Elisa protocols as described above. Hybridomas positive for the expression of immunoreactive MAbs were cloned at least twice by limiting dilution and MAb isotype analysis was performed. Purified MAb IgG was prepared from ascites fluid using protein-A affinity chromatography.

After fusion screening showed that immunization of mice with peptide #959 conjugated to KLH and screened in a solid-phase ELISA format with peptide #958 coupled to ovalbumin resulted in six positive parental signals (identified as 369.1 through 369.6). Both peptides have amino acids 35-42 of the βA4 region, different N-terminal spacers, and a cysteine for covalent coupling to carrier proteins (Figure 6). The free C-terminus with the charged carboxy group and a limited length of only 8 amino acids favors the generation of antibodies which are specifically directed against longer forms of βA4 peptides; shorter βA4 peptides ending before amino acid 42 would

thus not be recognized.

Figure 6 diagrams the structure of the immunogen (carrier-peptide) and the screening peptide (carrier-screening peptide) used.

Two of the six parental signals were ultimately not clonable. Of the remaining four, two gave immunoprecipitation/scintillation signals only a few percent above normal non-immune controls; the other two (identified as 369.6 and 369.2) showed signals of 18% and 19% respectively. Production of monoclonal antibodies from ascities fluid and subsequent immunopurification of these clones was done. Table I compares the data obtained with the IPSA for hybridoma supernatants and purified antibodies.

Table I

Cell Line	Isotype	IPSA (supernatant)	IPSA (purified)
369.1	IgG1/IgG2b	3%	N.D.
369.2	IgG1	19%	25% (with 5 µg)
369.3	IgG1	2%	N.D.
369.6	IgG2b	18%	7% (with 10 µg)

Table 1. Comparison of antibody activities in hybridoma cell lines. IPSA data represents the percent of in vitro translated βA4 which could be immunoprecipitated by either hybridoma supernatants or purified antibody.

Example 5: Immunoprecipitation/Scintillation Assay for Hybridoma Screening

To develop and screen for monoclonal antibodies which recognize the βA4 peptide in solution rather than when attached to a solid phase, an assay was developed in which immunoprecipitation of an ^{35}S-methionine-labelled in vitro-translated βA4 (IVT βA4) is measured. A standard amount of in vitro translated βA4 is allowed to form antibody/antigen complexes in a solution which can be optimized for ionic

strength, pH, and detergent composition. After the immune complexes are precipitated with Protein G (Omnisorb cells) and washed extensively, bound radioactivity is counted in a liquid scintillation counter; background is subtracted and the efficiency of precipitation calculated. This Immunoprecipitation/Scintillation assay (IPSA) allows for both the rapid identification and characterization of antibodies, and has been used to test a variety of βA4 antibodies. The assay can be applied in general to monoclonal hybridoma supernatants as well as polyclonal sera to identify antibodies which can be used for immunoprecipitations. Typically 18 IPSAs can be performed in one day. This is therefore a fast and informative secondary hybridoma screening method

Briefly, approximately 1.5 x 10^5 DPMs of ^{35}S-methionine-labeled in vitro-translated βA4 (IVT βA4) were added to 10 µl of a 10x immunoprecipitation buffer (150mM NaCl, 10%NP-40, 5% deoxycholic acid, 1% SDS, 500mM Tris pH8). To this, 90 µl of monoclonal cell supernatant from the monoclonal fusion of interest (our designation # 369) were added and allowed to react for 2 hrs at 4°C. Background was determined using 90 µl of supernatant of a non-reactive clone; the positive control was 90 µl of supernatant containing monoclonal antibody 286.8A which was made previously against a crude synthetic βA4(1-42) preparation. After 2 hrs, 40 µl of a 10% solution of Omnisorb cells (Calbiochem) equilibrated in 1x immunoprecipitation buffer (RIPA buffer; 150mM NaCl, 1%NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH8) were added and allowed to react for an additional 2 hrs at 4°C with rocking. The cells were pelleted by centrifugation for 5 min at 4500 g and 4°C, and washed 3x with 800 µl cold 1x immunoprecipitation buffer. Pellets were quantitatively transferred to scintillation vials and counted in a Beckman LS6000 scintillation counter in the Auto DPM mode. The percentage of βA4 immunoprecipitated was calculated.

Immunoprecipitation/Scintillation assays were performed with 1 µg of purified monoclonal antibody 369.2B in a total volume of 100 µl 1x immunoprecipitation buffer to which 5 µg of competing peptide were added. Incubations and precipitations

were as described above.

Figure 7 depicts the percentage of immunoprecipitated IVT βA4 as a function of antibody concentration for MAbs 369.2, 369.6 and MAb 286.8A. Under the conditions of this assay, 369.2 (and further subclone 369.2B) is approximately four times better than 369.6 in immunoprecipitating soluble IVT βA4, but precipitates a little less than half as much as 286.8A. Figure 7 shows the results of Immunoprecipitation of in vitro translated βA4 vs. antibody concentration (μg antibody/100 μl RIPA buffer) where;

$$\text{Percent βA4 immunoprecipitated} = \frac{(\text{dpms with MAb}) - (\text{dpms with non or preimmune control})}{(\text{total βA4 dpms/reaction})}$$

Percents with a given MAb concentration varied only a few percentage points between and within experiments.

IPSA for Monoclonal Characterization

Approximately 1.5 x 10^5 DPMs of ^{35}S-methionine-labeled in vitro-translated βA4 were added to various amounts of purified monoclonal antibody, either 369.2B, 369.6, or 286.8A, in a total volume of 100 μl 1x immunoprecipitation buffer, and allowed to react as described above. Immune complexes were precipitated with Omnisorb, washed, and counted as described above.

Example 6: Characterization of MAb 369.2B

To further characterize the best cell line, 369.2B, a competition immunoprecipitation/scintillation assay (Competition IPSA) was performed. In this variation 369.2B was added to an approximate 200 fold molar excess of unlabelled competitor peptide at the same time as labelled in vitro translated βA4 1-42. As expected, peptides to the human βA4 region, 1-40, 1-11, 1-28, 12-28, as well as the reverse peptide 40-1 did not compete with the ^{35}S-methionine-labeled in vitro-translated βA4 for immunoprecipitation, whereas the complete 1-42 peptide did

(Figure 8).

These results were corroborated in a solid-phase ELISA format: the absorbed ovalbumin-coupled screening peptide which contains the βA4 region 35-42, as well as the 1-42 peptide, compete whereas 1-40 did not (Figure 9). The decreased competitiveness of the 1-42 peptide compared to the ovalbumin coupled 35-42 may be due to conformational and/or solubility factors involving the antigenic determinant, or perhaps more simply to the particular stoichiometry of the conjugation (ovalbumin, a carrier with molecular weight of 45 kD compared to 4 kD for the 1-42 peptide, and having somewhere between 5-15 conjugatable maleimide groups per mole of carrier).

Figure 8 shows Immunoprecipitation Peptide competition/Scintillation Assay for epitope determination of MAb 369.2. Peptide competitor (5 µg) was mixed with in vitro translated βA4 (~1.5 x 10^5 DPMs or ~200 pg) then immunoprecipitated with 2 µg 369.2, where;

$$\text{Percent } \beta A4 \text{ immunoprecipitated} = \frac{(\text{dpms with MAb}) - (\text{dpms with non or preimmune control})}{(\text{total } \beta A4 \text{ dpms/reaction})}$$

Percents with a given MAb concentration varied typically only a few percentage points between and within experiments.

Figure 9 shows epitope mapping of MAb 369.2 by competitive assay. C369.2 (50ng IgG/100 µl) was preincubated with or without synthetic competitor peptides (22°C, 1 hr), then subjected to Elisa against plate-bound Ovalbumin-coupled 35 - 42 (200ng/well). Percent competition was calculated relative to MAb binding in the absence of competitor, i.e. where;

$$\%\ \text{competition} = \frac{(\text{signal w/o competitor}) - [(\text{signal w competitor}) - (\text{background})]}{(\text{signal w/o competitor})}$$

The solid square is 35-42(OVA) peptide conjugate; the open square is 1-42 peptide; and the solid diamond is 1-40 peptide.

From this data we conclude that monoclonal 369.2B is specific for the C-terminal end of the full length (1-42) βA4 peptide. Although the exact antigenic

determinant has not been fine mapped, it clearly involves residues beyond position 40 and, since the antibody was made to a short synthetic peptide the determinant is unlikely to involve other residues of βA4 which may be conformationally juxtaposed. Specifically, 369.2B is a very important tool in recognizing βA4 species ending at position 42.

One additional and interesting observation from the peptide competition assay is the enhanced immunoprecipitability of in vitro-translated βA4 by the decapeptide 25-35. This phenomena has also been seen in assays using one other monoclonal antibody (i.e., 286.8A) as well as one rabbit polyclonal antisera (data not shown). We also know from other experiments using varying amounts of detergent, specifically SDS, in IPSA assays with MAb 286.8A, that more βA4 can be immunoprecipitated with increasing amounts of detergent (data not shown). SDS, interestingly, has been shown to be ineffective in solubilizing βA4 aggregates, at least as shown by SDS-PAGE (Burdick et al., 1992). However it is not immediately clear why SDS should enhance the immunoprecipitability of βA4.

Example 7: Immunohistochemical Studies

We have undertaken immunohistochemistry studies with 369.2B. The staining pattern of 369.2B (1/10,000 dilution of a 22 mg/ml ascities purified antibody solution) when compared to the monoclonal antibody 286.8A which we have shown recognizes epitope 3-8 of βA4 and is human specific (data not shown) showed interesting differences. Results obtained from immunohistochemistry demonstrated that 369.2B is an excellent antibody (at 1/10,000 dilution) to specifically label amyloid plaques cores, diffuse as well as fibrillar amyloid deposits and vascular amyloid deposits (Figure 10).

Figure 10 is a photomicrograph showing β-amyloid plaques, blood vessels, and perivascular deposits of βA4 in a paraffin embedded 10 μm thick section from the brain of a 76 year old female patient with Alzheimer's disease. Tissue sections were

pretreated with 88% formic acid (30 min), then labeled using an avidin-biotin-peroxidase kit (Vector Laboratories, Burlington, CA) with nickel-diaminobenzidine as the chromagen. Monoclonal antibody 369.2B labels plaques with a variety of morpologies, including cored, perivascular, and diffuse (non-amyloidotic) plaques. It also labels a subset of amyloidotic blood vessels.

Further studies also showed that βA4 1-43 peptide was not able to compete for staining (more than a 1000 fold excess peptide), whereas βA4 1-42 completely abolished the signal (Table 2). Again as expected, 1-40 or 40-1 did not compete for staining. From these studies we can already conclude that this antibody is an excellent tool for immunohistochemistry. As suggested by in vitro studies which show physico-chemical differences between 1-40 and 1-42, it is possible that these two βA4 species show distinct patterns in Alzheimer brains. With the monoclonal antibody of the instant invention, we are now able to begin addressing this question. Thus the monoclonal antibody and methods of the instant invention are useful for diagnostic and therapeutic uses in all immunological and related methodologies which can be applied to the detection, monitoring, extraction, inhibition and modification of unique βA4 species, in the diagnosis and treatment of AD.

Table 2

	Monoclonal Antibody used for Staining	
Competing peptide	N-terminal Mab 286.8A	C-terminal Mab 369.2B
None/buffer	+++	+++
None/DMSO	+++	+++
Human "40-1"	+++	+++
Human "1-16"	-	+++
Mouse "1-16"	+++	+++
Human "1-40"	-	+++
Human "1-42"	-	-
Human "1-43"	-	+++
Human "35-42" with spacer	+++	-

Table 2. Results from competition binding experiments and inhibition of staining, a +++ indicates strong staining, - indicates no detectable staining.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: König, Gerhard
Graham, Paul

(ii) TITLE OF INVENTION: Monoclonal Antibody Specific for BA4 Peptide

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Allegretti & Witcoff, Ltd.

(B) STREET: 10 South Wacker Drive Suite 3000

(C) CITY: Chicago

(D) STATE: Illinois

(E) COUNTRY: USA

(F) ZIP: 60606

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McDonnell, John J

(B) REGISTRATION NUMBER: 26,949

(C) REFERENCE/DOCKET NUMBER: 95,216

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 312-715-1000

(B) TELEFAX: 312-715-1234

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Asp Gly Asp Gly Asp Met Val Gly Gly Val Val Ile Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Cys Gly Gly Gly Gly Gly Met Val Gly Gly Val Val Ile Ala
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 4..5

(D) OTHER INFORMATION: /label= Beta
/note= "Beta cleavage site in APP"

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 20..21

(D) OTHER INFORMATION: /label= Alpha
/note= "Alpha cleavage site in APP, residues 16/17
of BA4."

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 46..47

(D) OTHER INFORMATION: /label= Gamma
/note= "Gamma cleavage site in APP"

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 5..47
(D) OTHER INFORMATION: /label= BA4
/note= "BA4 peptide"

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 33..56
(D) OTHER INFORMATION: /label= Tm
/note= "Transmembrane region of APP"

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: 1..32
(D) OTHER INFORMATION: /label= Ex
/note= "N-terminal extracellular part of APP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val
 1               5                  10                  15
His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys
            20                  25                  30
Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val
        35                  40                  45
Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys
    50                  55
```

We claim:

1. A monoclonal antibody that binds βA4 peptide associated with diffuse amyloid, fibrillar amyloid, neurofibrillary tangles, and vascular amyloid, wherein said βA4 peptide contains a free carboxy terminal amino acid that corresponds with amino acid 42 of the βA4 peptide amino acid sequence.

2. A monoclonal antibody of claim 1 that recognizes the C-terminal amino acid 42 of the βA4 peptide (SEQ ID NO:3).

3. A monoclonal antibody of claim 1 that is an IgG class antibody.

4. A monoclonal antibody of claim 1 that is an IgG1 or IgG2b subclass antibody.

5. A monoclonal antibody that is identified as 369.2B, and is produced by the cell line deposited with the American Type Culture Collection (ATCC) as ascension number HB 11829.

6. An immunologically active fragment of the monoclonal antibody of claim 1.

7. An immunologically active fragment of the monoclonal antibody of claim 5.

8. A cell which is identified by the ATCC ascension number HB 11829.

9. A method of detecting the presence of βA4 peptide in tissue comprising contacting a tissue sample with monoclonal antibody of claim 1, and detecting the presence of monoclonal antibody.

10. A method of selectively purifying βA4 peptide comprising contacting a sample to be purified with the monoclonal antibody of claim 1, separating the βA4 peptide from the sample to be purified, and isolating the βA4 peptide.

11. A method of detecting of βA4 peptide associated with Altzheimer's Disease, comprising contacting a sample to be tested with the monoclonal antibody of claim 1 and detecting the presence of βA4 peptide.

12. A method of detecting the presence of βA4 peptide in tissue comprising contacting a tissue sample with monoclonal antibody of claim 5 and detetting the presence of monoclonal antibody.

13. A method of selectively purifying βA4 peptide comprising contacting a sample to be purified with the monoclonal antibody of claim 5, separating the βA4 peptide from the sample to be purified, and isolating the βA4 peptide.

14. A method of detecting βA4 peptide associated with Alzheimer's Disease, comprising contacting a sample to be tested with the monoclonal antibody of claim 5, and detecting the presence of βA4 peptide.

15. A method of preventing aggregation of βA4 peptide comprising administering the monoclonal antibody of claim 1.

16. A method of preventing aggregation of βA4 peptide comprising administering the monoclonal antibody of claim 5.

17. A means for detecting the presence of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 1.

18. A means for detecting the presence of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 5.

19. A means for preventing the aggregation of βA4 peptide comprising an immunologically reactive fragment of the monoclonal antibody of claim 5.

20. A method of generating the antibody of claim 1, comprising immunizing a mammal with the peptide CDGDGDMVGGVVIA (SEQ ID NO:1) conjugated to a suitable immunological carrier.

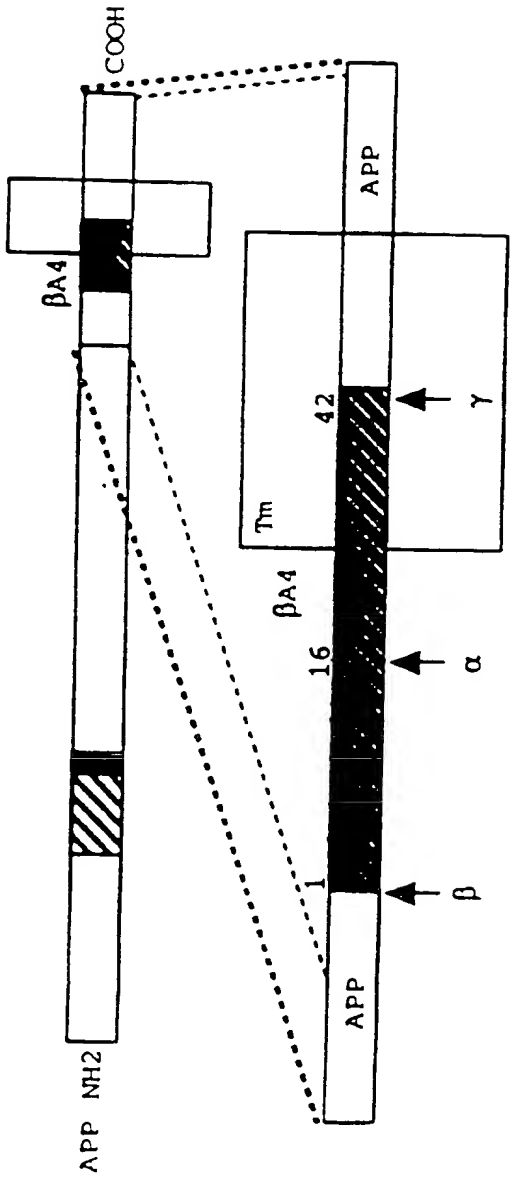


Figure 1

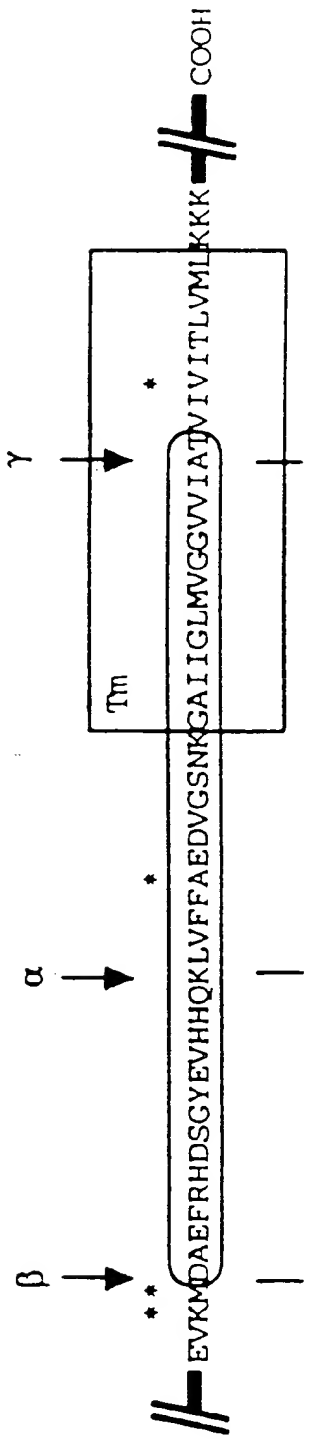


Figure 2

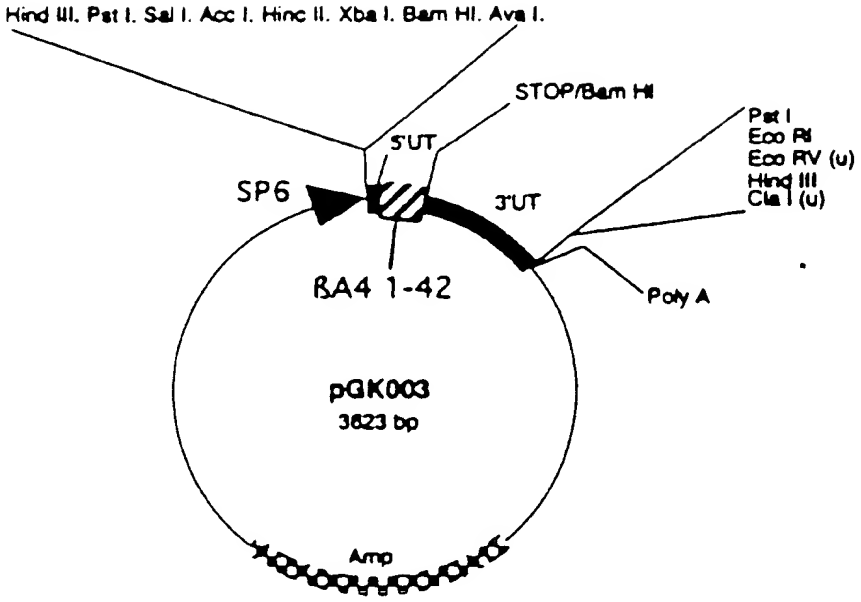

Hind III. Pst I. Sal I. Acc I. Hinc II. Xba I. Bam HI. Ava I.
STOP/Bam HI
5'UT
SP6
3'UT
Pst I
Eco RI
Eco RV (u)
Hind III
Cla I (u)
BA4 1-42
Poly A
pGK003
3623 bp
Amp

Fig. 3

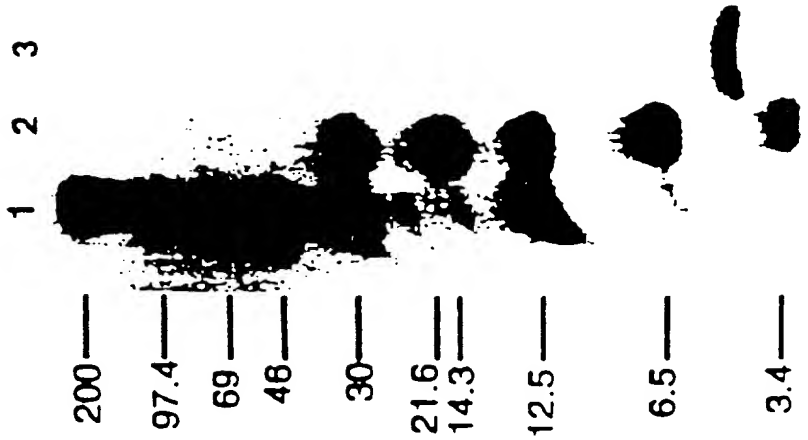

1
2
3
200
97.4
69
46
30
21.6
14.3
12.5
6.5
3.4

Fig. 4A.

1
2
3
200
97.4
69
46
30
21.6
14.3
12.5
6.5
3.4

Fig 4B.

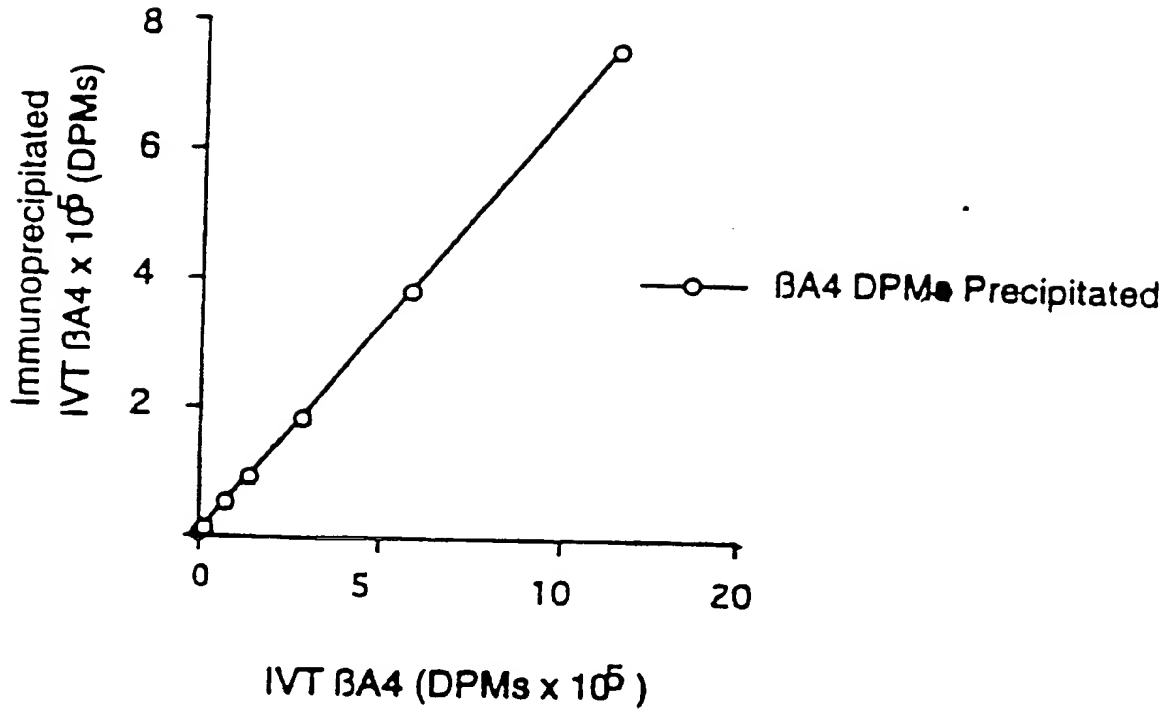
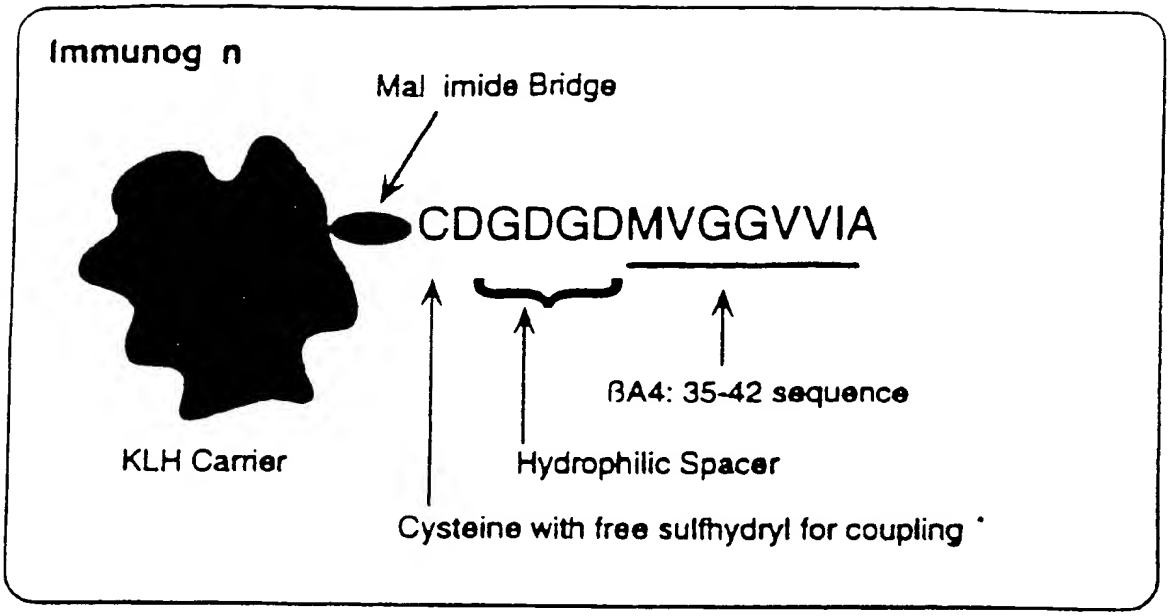

Immunoprecipitated
IVT βA4 x 10⁵ (DPMs)
8
6
4
2
0
5
10
20
IVT βA4 (DPMs x 10⁵)
βA4 DPMs Precipitated

Fig. 5


Immunog n
Mal imide Bridge
CDGDGDMVGGVVIA
βA4: 35-42 sequence
KLH Carrier
Hydrophilic Spacer
Cysteine with free sulfhydryl for coupling

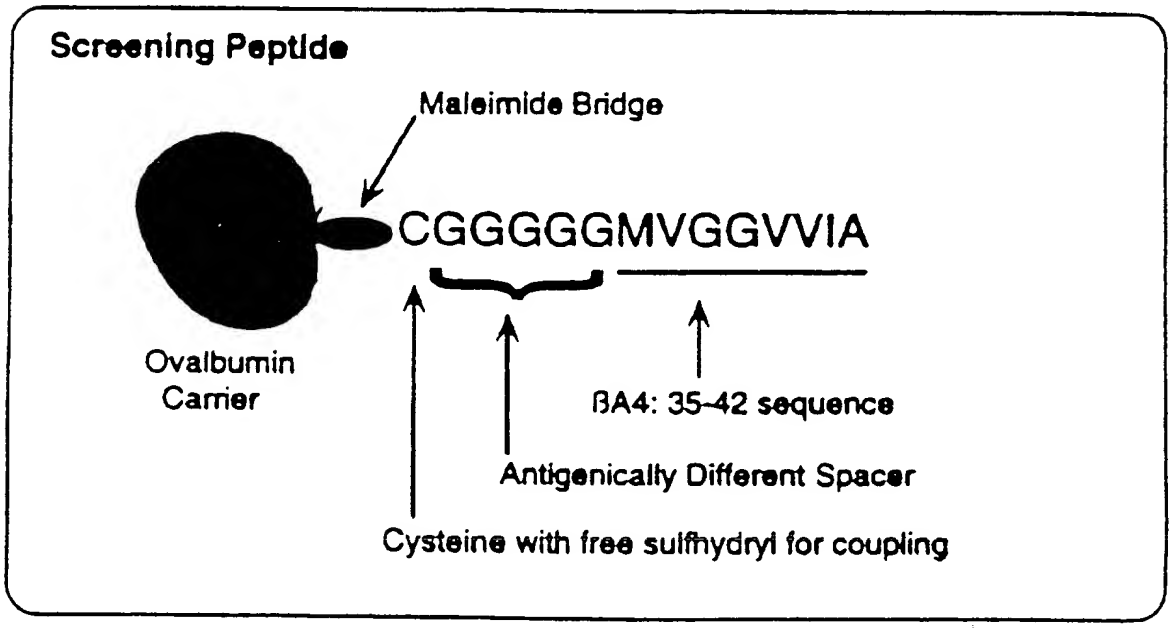

Screening Peptide
Maleimide Bridge
CGGGGGMVGGVVIA
Ovalbumin
Carrier
βA4: 35-42 sequence
Antigenically Different Spacer
Cysteine with free sulfhydryl for coupling

Fig 6

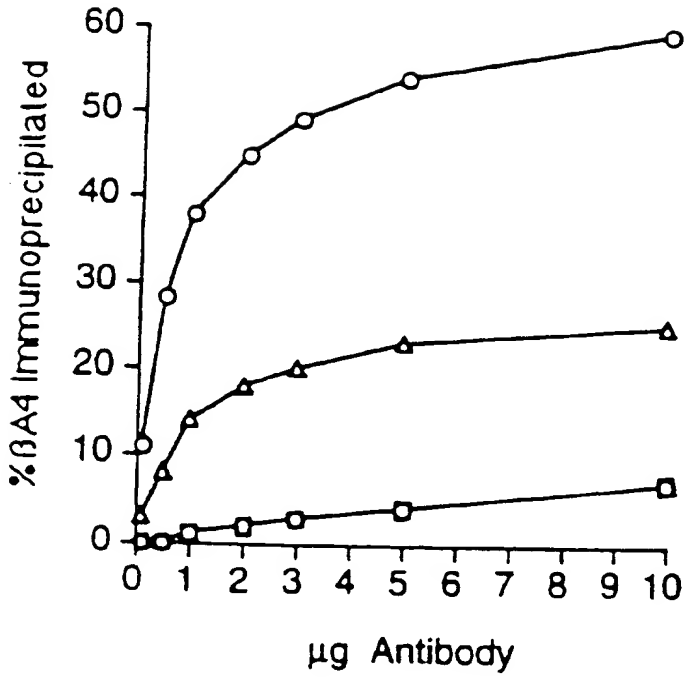

60
50
40
30
20
10
0
%βA4 Immunoprecipitated
0 1 2 3 4 5 6 7 8 9 10
µg Antibody
286.8A,
369.2B,
369.6

Fig. 7.

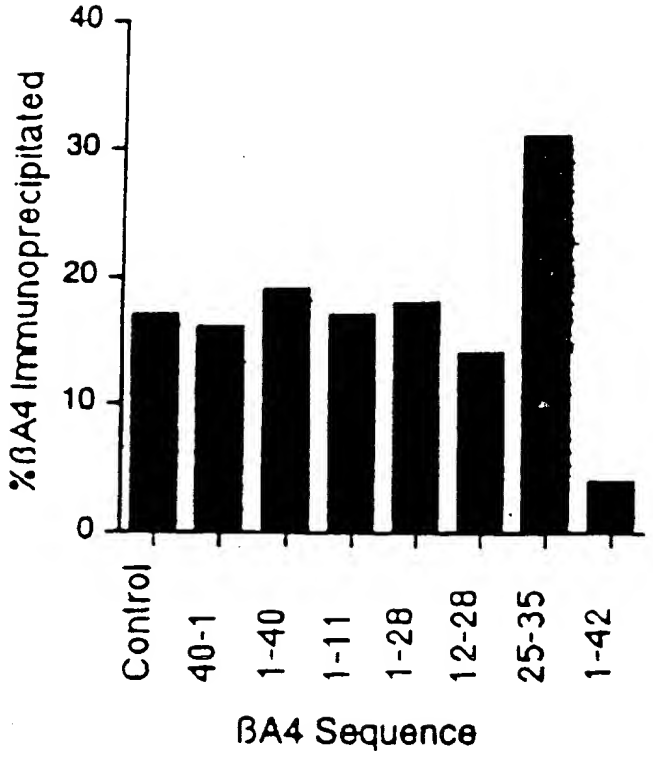

40
30
20
10
0
%βA4 Immunoprecipitated
Control
40-1
1-40
1-11
1-28
12-28
25-35
1-42
βA4 Sequence

Fig. 8.

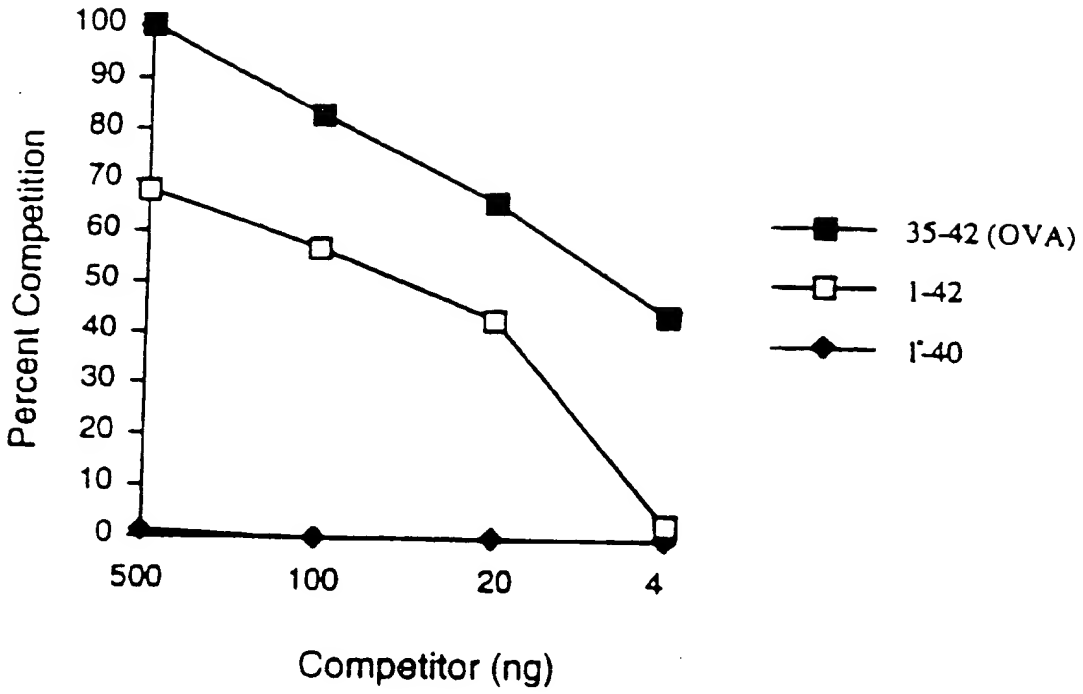

100
90
80
70
60
50
40
30
20
10
0
Percent Competition
500
100
20
4
Competitor (ng)
35-42 (OVA)
1-42
1-40

Fig. 9.

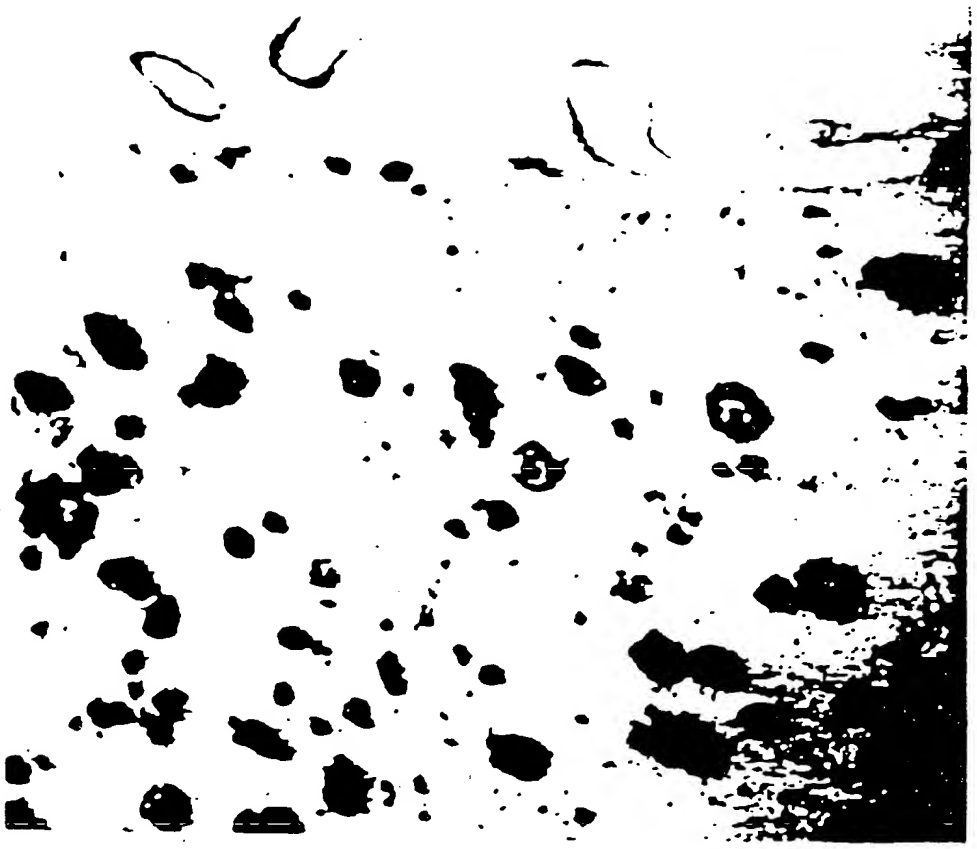


Fig. 10

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/02491

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 17197 (TAKEDA CHEMICAL INDUSTRIES LTD ;SUZUKI NOBUHIRO (JP); ODAKA ASANO) 4 August 1994	1-19
Y	& EP0683234 22 NOVEMBER 1995 (SEE PAGE 4 LINE 49 -PAGE 11 LINE 25) see the whole document	20

Y	AMERICAN JOURNAL OF PATHOLOGY, vol. 144, no. 5, May 1994, pages 1082-1088, XP000573948 GREER M. MURPHY ET AL.: "DEVELOPMENT OF A MONOCLONAL ANTIBODY SPECIFIC FOR THE COOH-TERMINAL OF BETA-AMYLOID 1-42 AND ITS IMMUNOHISTOCHEMICAL REACTIVITY IN ALZHEIMER'S DISEASE AND RELATED DISORDERS." cited in the application see the whole document	20

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 1996	25.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/02491

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 16628 (UNIV CALIFORNIA) 31 October 1991	

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/02491

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9417197	04-08-94	EP-A-	0683234	22-11-95
WO-A-9116628	31-10-91	EP-A-	0527823	24-02-93
		US-A-	5427931	27-06-95
		US-A-	5270165	14-12-93
		US-A-	5213962	25-05-93

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02491

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 15,16,19 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.